Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 93 DECEMBER, 1956 No. 3

SECTION MEETINGS

CLEVELAND, O.	
Univers.ty Hospital	October 15, 1956
DISTRICT OF COLUMBIA	
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ILLINOIS	
Michael Reese Hospital	October 9, 1956
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Ultra-Violet Flying Spot Television Microscopy. Study of Living HeLa Cells.* (22772)

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In another communication the authors have described a technic for obtaining relatively monochromatic U-V television images by means of the flying spot microscope(1). This system is in operation, and the purpose of this paper will be to report results of preliminary experiments designed to establish the period of time over which unchanged images can be obtained with the flying spot system.

Methods. The light source is a U-V emitting cathode ray scanner tube on the face of which is formed a 240 line raster. This raster is traced by a spot deflected horizontally at 60 cycles/second and vertically at one cycle every 4 seconds. These sweep rates were arbitrarily chosen to investigate potentialities of the system. A minified image of this raster is formed on the specimen by a Bausch and Lomb .75 NA Grey reflecting objective. Transmitted radiant energy is collected by a reflecting condenser optically matched to the objective and is focused on the entrance slit of a Bausch and Lomb grating monochromator. An ultra-violet-sensitive photomultiplier tube is located at the exit slit of the monochromator. The monochromator is adjusted to 2680 Angstroms with a 100 Angstrom band width. The electrical output of the multiplier tube is amplified and modulates a monitor display tube. The time bases of this monitor tube are locked in synchronism with those of the scanner tube. The screen has a very long persistency, of the order commonly employed for radar tubes. This long persistency screen is necessary because of the long vertical sweep cycle. Thus, the entire system becomes an

* Supported by Damon Runyon Memorial Fund for Cancer Research, Rockefeller Foundation, Texas Instruments—GSI Foundation, Caruth Foundation, Southwestern Medical Foundation and private citizens of Dallas.

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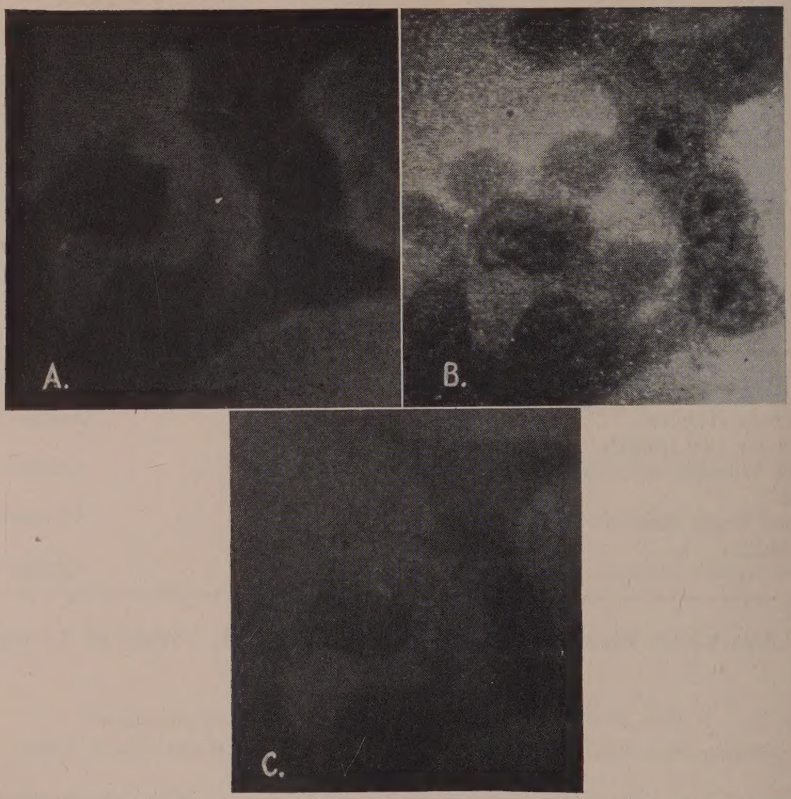

FIG. 1. Living HeLa cells at 2680 Å (667×). A. Original appearance. B. presence of cytoplasmic appearance at the end of 6 hr of continuous scanning. C. Return to normal after 12 hr interruption in scanning.

image converter and a specimen scanned in ultra-violet is displayed in visible light. The image on the monitor screen is photographed by a Polaroid Land camera. The signal to noise ratio of the display at these low radiation levels requires that the images of several consecutive frames be stored in the film emulsion. The signal to noise ratio then becomes a function of the square root of the number of frames stored. During the entire course of these experiments the microscopic field was continually irradiated by the scanner tube and photographs were obtained at periodic intervals from 15 minutes to 1 hour. In the experiments described, living HeLa strain cells were prepared on Vycor cover slips and sealed to Vycor slides by means of a wax vaseline ring. The cells were suspended in their own media composed of chick embryo extract, human ascitic fluid and Hank's balanced salt solution or in ascitic fluid alone, or in balanced salt alone. We were unable to detect any significant differences in the images obtained attributable to the nature of the media employed. Once the preparation was mounted on the microscope it was allowed to remain at room temperature (72°F) for the duration of the experiment.

Results. Fig. 1A shows a typical absorption photograph of living HeLa cells at 2680 Å as obtained with the flying spot technic. This field was then continuously irradiated and intermittent photographs of the monitor tube were obtained. The photographic images remained the same for a period of approximately 6 hours. At this time cytoplasmic bubbles began to form about the periphery of some of the cells in the field. These bubbles

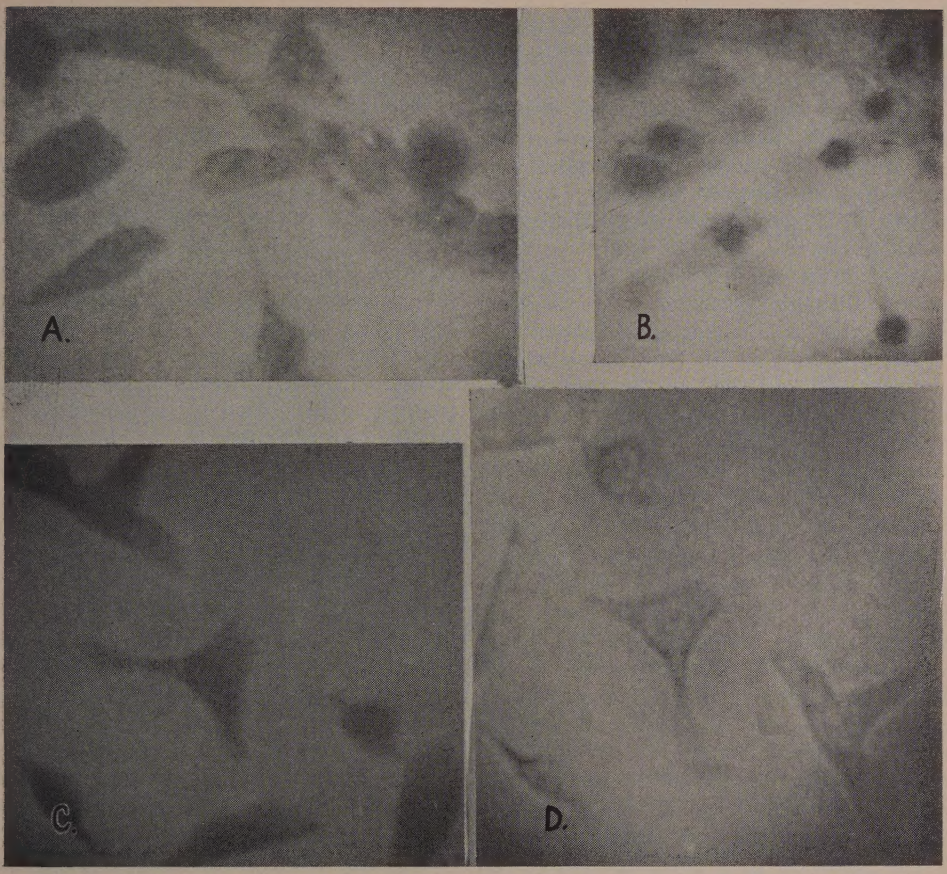

FIG. 2. Living HeLa cells at 2680 Å (533×). A. Original appearance. B. Following 6 hr of continuous scanning showing marked nuclear opacity and cytoplasmic transmission. C. Living HeLa cells at 2680 Å (533×). D. Living HeLa cells at zero orders showing lack of typical U-V absorption.

showed absorption at 2680 Å as may be seen in Fig. 1B but they were clear when viewed with visible light. In this experiment the phenomenon was reversible following a 12 hour respite from the continual irradiation, and the bubbles did not reappear upon subsequent irradiation of 3 hours duration as illustrated by Fig. 1C. One notes that the cytoplasmic absorption, nuclear lack of absorption, nuclear membrane and nucleolus all remain constant in their absorption appearance during this prolonged exposure to U-V irradiation. These 3 photographs do not show identical background densities of the photographic plates in each case and the density of the absorption is thus not quantitatively comparable. The present instrumentation does not permit a constant monitor screen brightness over 64 frames, thus variations in the amount of light reaching the photographic plate are inevitable. In a longer experiment consisting of 7 hours of continuous irradiation marked changes in the absorption pattern of the cells occurred at the end of the 7 hour period. These changes consisted of a complete opacity of the nucleus with lack of definition of the nucleolus, and a relative lack of the normal cytoplasmic absorption. The contrast between the appearance of these cells at the end of 2 hours and at the end of 7 hours is illustrated by Fig. 2A and 2B. In this experiment the cytoplasmic bubbles

described in the previous experiment appeared at the end of 5 hours and disappeared just before the appearance of the nuclear opacity.

In a third experiment continuous irradiation was applied to the cells for 6 hours. During this time the photographic images of the monitor screen remained the same. In this experiment 2 photographs were obtained at each time interval. Fig. 2C is a photograph obtained at the end of 2 hours. In this photograph the monochromator is set at 2680 Å. Fig. 2D is a photograph obtained immediately after Fig. 2C, the monochromator in this case is adjusted to the zero orders. Fig. 2C is a typical absorption photograph while Fig. 2D illustrates an image formed principally by light scattering. The latter image shows the characteristic clarity of the living nucleus when viewed with visible light.

Discussion. The principle of the flying spot microscope as described by Roberts and Young(2) has been adapted for use in the deep ultra-violet. The purpose of this adaptation was to find if possible a method which would permit the continuous or nearly continuous recording of ultra-violet absorption images of living cells without producing severe irradiation damage in the process. It would appear from these preliminary studies that this technic will yield just such results.

The failure of each group of cells to respond in an identical fashion to roughly equivalent time intervals of continuous scanning is not explained. It may represent differences in culture technic as the cells were transferred from stock cultures at irregular intervals, or it may represent variations in amount of irradiation. Complete regularity of U-V emission from the scanner tube is not possible in the present state of tube and phosphor development. Furthermore, day to day variations in the room temperature and instruments settings could not be rigidly controlled and it is conceivable that minor uncontrolled variations contributed to the experimental variations observed.

The data presented here are in agreement with those presented by Brumberg and Larionow(3). These authors describe the undamaged nucleus to show little or no absorption at 2680 Å. This is in marked contrast to Caspersson's observations(4), and may perhaps be accounted for by the lack of irradiation damage in this and Brumberg's work. Thus, lack of nuclear absorption by visible or ultra-violet light in normal living cells is one criterion of the absence of significant irradiation damage. As irradiation damage occurs the nuclear absorption increases until the nucleus becomes homogeneously opaque. The apparent increasing density of the nucleus in contrast to its normal transmissiveness may represent an alteration in the physical state of the DNA of the chromosomes. Such alterations have been produced by Ris and Mirsky (5) by physiological saline or fixation procedures. They consider the appearance of structure in the nucleus in visible light to be good evidence for cellular damage.

It appears that the flying spot scanning system of U-V microscopy is capable of producing continuous absorption images of living cells without producing significant irradiation damage. The system may be further improved by several technics, the most important of which will be to make the spot monochromatic before it reaches the specimen.

Summary. 1. Preliminary experiments with the continuous production of monochromatic ultra-violet absorption images of living cells indicate that this may be accomplished by the flying spot television microscope technic.

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Received May 22, 1956. P.S.E.B.M., 1956, v93.

Inhibition of DOPA Decarboxylase by Aromatic Acids Associated with Phenylpyruvic Oligophrenia. (22773)

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It has been demonstrated(1) that the biochemical lesion associated with mental deficiency in phenylpyruvic oligophrenia is a failure of an enzyme system in the liver of these patients to hydroxylate phenylalanine to tyrosine. This condition results in the appearance of abnormal oxidation products of aromatic amino acids in the blood and urine(2). The light hair and skin color associated with this disease has suggested an impairment of tyrosine metabolism. Experimental support for this suggestion has recently been brought forth in the report, which shows that phenylalanine and the related aberrant aromatic acids associated with this disease, competitively inhibit plant as well as mammalian tyrosinase*(3). Further the repigmentation which has been observed when these patients are placed on a low phenylalanine diet(4) is consistent with this notion.

The recent finding that patients suffering with this affliction have low epinephrine plasma values(5) suggests a further interference with enzyme systems as a consequence of the appearance of abnormal amounts of phenylalanine and its related metabolites. These low plasma levels of epinephrine led us to examine the effects of these aromatic acids on the beef adrenal medulla DOPA decarboxylase, an enzyme which functions in the metabolic pathway for epinephrine synthesis.

Methods. DOPA decarboxylase was obtained from beef adrenal medulla as described by Langemann(6). The tissue was ground with sand in a mortar, suspended in an equal amount of 0.1 M phosphate buffer, pH 6.8 and centrifuged. The rate of decarboxylation was determined with a Warburg respirometer at 37°C. The gas phase was nitrogen. The main compartment contained 0.8 ml enzyme, 40 γ pyridoxal-5-phosphate and inhibitor. One side arm contained 20 μmoles D-L DOPA while the other contained 3.6 N H_2SO_4. After the removal of the air with nitrogen the vessels were closed and equilibrated for 10 minutes. The substrate was tipped into the main compartment and readings were made at 5 and 10 minute intervals. After 10 minutes the acid was tipped in, and a final reading was taken. Sodium phenylpyruvate, phenyllactic acid, and L-phenylalanine were obtained from Nutritional Biochem. Inc., phenylacetic acid was obtained from Matheson, Coleman and Bell Inc.; pyridoxal-5-phosphate was obtained from Mann Biochem. Inc., and D-L dihydroxyphenylalanine was obtained from Eastman Kodak Co.

TABLE I. Inhibition of Adrenal Medulla DOPA Decarboxylase by Phenylketonuria Metabolites.

Additions	Conc., μmoles/3 ml	μl CO_2/hr	% inhibition
Control	—	160	—
Phenylpyruvic acid	100	0	100
	50	0	100
	10	36	77
Phenyl-lactic acid	100	0	100
	50	36	77
	10	82	50
Phenylacetic acid	100	80	50
	50	163	0
L-phenylalanine	100	164	0

Each flask contained 800 mg adrenal medulla, 40 γ pyridoxal phosphate. Phosphate buffer pH 6.8. The gas phase was nitrogen.

Results. The results of these experiments on the inhibition of adrenal DOPA decarboxylase are given in the Table. Phenylpyruvate and phenyllactate were the most effective inhibitors of the series, while phenylacetic acid was poor. L-phenylalanine showed no inhibitory effect. It is interesting to compare these results with those obtained by Hartman *et al.*(7) in their study using DOPA decarboxylase from hog kidney cortex. They obtained comparable inhibitions of their enzyme with phenylpyruvate, and phenylacetic acid, but obtained no inhibitions with phenyllactate.

* To be published.

These data suggest an explanation for the low plasma levels of epinephrine in patients suffering from phenylpyruvic oligophrenia(5) in that these aromatic acids which accumulate as a result of biochemical lesion may inhibit the synthesis of epinephrine in the adrenal gland.

The importance of epinephrine in normal nerve function is yet to be elaborated; however, its presence in brain tissue is well established(8). Weil-Malherbe has shown that epinephrine plasma levels were significantly lower in a group of mental defectives than in a group of mixed patients(5). As yet, information is not available which would allow us to speculate on the influence of a deficiency of epinephrine on the etiology of mental deficiency.

Summary. Phenylpyruvic acid, phenyllactic acid and phenylacetic acid inhibit beef adrenal medulla DOPA decarboxylase. The implications of these data in phenylpyruvic oligophrenia are discussed.

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Received June 21, 1956. P.S.E.B.M., 1956, v93.

Stability of the Activator of Bovine Plasminogen. (22774)

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Mullertz and Lassen(1) first reported that a mixture of streptokinase and human plasminogen acquired a characteristic which neither the streptokinase nor the plasminogen exhibited alone, namely, the ability to activate bovine plasminogen. They concluded that bovine blood lacks a factor (proactivator) which is present in human blood and suggested that this proactivator combines with streptokinase to form an activator of bovine plasminogen. The studies of Troll and Sherry(2) and Sherry(3) indicated that the activator of bovine plasminogen was also the activator of human plasminogen. It was further demonstrated(2) that the activator which appears after adding streptokinase to human plasminogen was lost by acidification and regained by the re-addition of streptokinase after neutralization of the fraction, indicating that streptokinase was required for activator activity.

This communication calls attention to the observation that a loss of activator can occur during the incubation of a mixture of streptokinase and human plasminogen.* The rate of disappearance of the activator has been found to be affected by the duration of incubation, the initial streptokinase concentration and the particular plasminogen preparation. These observations have significant bearing on the interpretation of the *in vitro* and *in vivo* activation of plasminogen by streptokinase.

Materials. Streptokinase (SK), Varidase (Lederle) was dialyzed before use. Bovine plasminogen was made according to Milstone (4) and then lyophylized. Lysine ethyl ester (LEE) was synthesized according to Werbin and Palm(5) and stored over phosphorous

* During preparation of this manuscript, Mullertz (*Biochem. J.*, v61, p424, Nov. 1955) reported on similar observations.

pentoxide. Several fractions of human plasminogen were prepared. The preparations were as follows: #1) A euglobulin precipitate, made from placental blood according to Milstone(4), #2) A lyophylized pooled sample of human plasma, #3) A pH 2.5 acid extract of placental Fr. III_2, prepared as described by Christensen(6) and further purified by an adsorption-elution technic,† #4) A Fr. III, obtained as described by Oncley *et al.*(7). These reagents were kept in a desiccator and weighed out immediately before use. A pH 6.4, 0.1 M phosphate buffer was prepared by mixing 300 ml of 0.1 M Na_2HPO_4 with 700 ml 0.1 M NaH_2PO_4, and a pH 7.15 0.1 M phosphate buffer, by mixing 700 ml 0.1 M Na_2HPO_4 and 300 ml of 0.1 M NaH_2PO_4.

Methods. Plasmin, the proteolytic enzyme resulting from the activation of plasminogen, exhibits lysine ethyl esterase activity. Thus, when L-lysine ethyl ester (LEE) is incubated with plasmin, lysine is formed(8). 'Activator' is measured by incubating mixtures of SK and human plasminogen with excess bovine plasminogen and LEE. The amount of lysine liberated under these conditions serves as a measure of the concentration of bovine plasmin, which, in turn, is related to the concentration of activator. The reagents are prepared as follows: the SK, human plasminogen and bovine plasminogen are prepared in pH 6.4 0.1 M phosphate buffer, the SK and human plasminogen at concentrations indicated in the text, the bovine plasminogen, at 4%. A 0.168 M solution of LEE hydrochloride is prepared just prior to use in pH 7.15, 0.1 M phosphate buffer. One ml of each of the 4 reagents is added to the reaction flask; the resulting pH of the reaction mixture is 6.4. Enzyme blanks are prepared by replacing the LEE solution with pH 6.4 buffer, and a substrate blank, by replacing the mixture of SK, human plasminogen and bovine plasminogen with pH 6.4 buffer. The order of addition of reagents is described under each experiment. In certain experiments one or more of the protein components were omitted from the reaction mixture and their blanks in order to establish adequate controls. These are described in each experiment. The tubes are incubated at 37° for 60 minutes. Aliquots are then removed and the lysine determined by a modification(9) of the Spies and Chambers method(10). The results are reported as % hydrolysis of LEE.

† Unpublished data.

TABLE I. Effect of Streptokinase Concentration on Activation of Bovine Plasminogen.

Streptokinase in reaction mixture, units	% hydrolysis of LEE	
	Without bovine plasminogen	With bovine plasminogen
25,000	3.5	45.5
12,500	3.0	41.5
6,250	2.0	31.0
3,125	1.0	24.5

Results. 1) *Effect of streptokinase concentration on ability of a mixture of streptokinase and human plasminogen to activate bovine plasminogen.* Reaction mixtures with and without bovine plasminogen and containing 0.06 mg N of human plasminogen #1, LEE and varying amounts of SK were prepared by mixing the reagents in the above order. The results (Table I) show that with a constant amount of human plasminogen the ability to activate bovine plasminogen increases with increasing SK concentration.

2) *Effect of human plasminogen concentration on ability of a mixture of streptokinase and human plasminogen to activate bovine plasminogen.* This experiment was carried out with the same reagents and order of addition as used in Exp. 1. In this case, however, the SK concentration was kept constant at 6,250 units while the concentration of human plasminogen was varied. The results (Table II) demonstrate that the ability to activate bovine plasminogen is also dependent on the

TABLE II. Effect of Human Plasminogen Concentration on Activation of Bovine Plasminogen.

Human plasminogen reaction mixture, mg	% hydrolysis of LEE	
	Without bovine plasminogen	With bovine plasminogen
.10	2.0	47.0
.08	2.0	31.5
.06	2.0	31.0
.04	.0	22.5
.02	.0	6.0

TABLE III.* Effect of Preincubation Time on Ability of a Mixture of Streptokinase and Human Plasminogen to Activate Bovine Plasminogen.

Pre-incubation mixture	Time added, min.	Added to	% hydrolysis of LEE
(a) SK + human plasminogen	2	Bovine plasminogen and LEE	56
	30		39
	45		35
	60		31.5
	90		22

* Conditions described in text.

concentration of human plasminogen.

3) *Effect of preincubation time on ability of a mixture of streptokinase and human plasminogen to activate bovine plasminogen.* A mixture containing 1000 units of SK and 0.35 mg N of human plasminogen #3/ml was incubated at 37°. For assay, 1 ml aliquots were removed at intervals and added to freshly prepared solutions containing bovine plasminogen, LEE and buffer. The data are shown in Table III.

A gradual loss of ability of the mixture of SK and human plasminogen to activate bovine plasminogen is evident; SK and human plasminogen incubated separately were stable.

4) *Effect of addition of streptokinase or human plasminogen on a partially inactivated mixture of streptokinase and human plasminogen.* A mixture containing 200 units of SK and 0.6 mg N of human plasminogen #2 per ml was incubated at 37° and at the stated intervals samples were removed for assay. When partial inactivation had occurred (60 min.—see Table IV), 1 ml aliquots were removed from the solution. The first was used as a control; 0.6 mg N of human plasminogen #2 was added to the second; 200 and 1000 units of SK were added to the third and fourth, respectively. Each aliquot was then assayed for 'activator.' The results are tabulated in Table IV.

TABLE IV.* Effect of Streptokinase and Human Plasminogen on a Partially Inactivated Mixture.

Pre-incubation mixture	Time added, min.	Added to	% hydrolysis of LEE
SK + human plasminogen	2	Bovine plasminogen and LEE	61
	30		53.5
	45		49.5
	60		42
(1) Control	—	"	42
(2) Human plasminogen (.6 mg N)	2		47
(3) 200 SK	2		54
(4) 1000 SK	2		61

Component added to 60 min. preincubation mixture.

* Conditions described in text.

The results indicate that although some increase in the concentration of activator may have occurred on addition of human plasminogen (see also Table II), a greater increase was observed when SK was added, suggesting that the loss of SK may be the cause of the decreased ability of the mixture to activate bovine plasminogen.

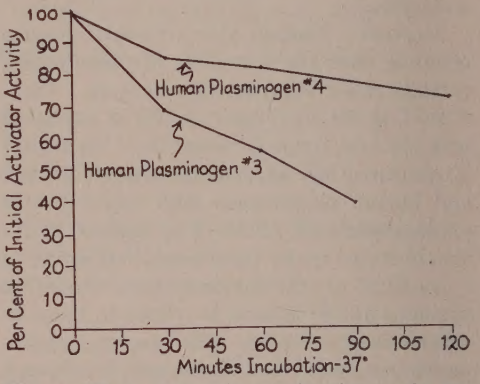

FIG. 1. Effect of plasminogen fraction on the stability of activator.

5) *Effect of human plasminogen preparation on the loss of the activator.* It was predetermined by activator assay that a 50-60% hydrolysis of LEE was obtained by a mixture of 1000 u SK and either 0.35 mg N of plasminogen #3/ml or 0.1 mg N plasminogen #4/ml. These mixtures were incubated at 37° and tested periodically for activator content. The rate of inactivation (Fig. 1) is shown to vary with the human plasminogen fraction employed.

6) *Effect of SK concentration on the loss of activator at pH 7.2.* Since the rate of inactivation could have physiological significance, it was decided to study the effect of SK concentration on the rate of disappearance of the 'activator' at physiological pH. The re-

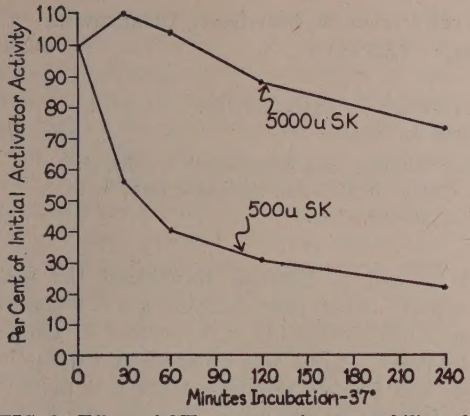

FIG. 2. Effect of SK concentration on stability of activator.

sults were essentially the same as those obtained at pH 6.4. The results (Fig. 2) show that with a constant amount of human plasminogen the rate of decrease of activator is greater at the lower SK concentration.

Discussion. The data presented are interpreted to indicate that SK may be consumed by a "deterioration route" as well as by the route in which it activates plasminogen. It can not be established from these experiments whether the deterioration of activator in a mixture of SK and human plasminogen occurs due to the loss of SK or to the loss of the formed activator, or both. Since variations in SK concentration as well as plasminogen concentration can affect not only the quantity of activator formed but also the rate at which deterioration of activator occurs, the relative rates at which these reactions occur present an obstacle to the study of the kinetics of the activation of plasminogen by SK.

Some speculation on the effects of *in vivo* administration of SK appears to be in order. If SK undergoes reactions *in vivo* as are described herein *in vitro,* its administration to humans(11) could result in variable activation of plasminogen depending on the mode and rate of administration of a given dose of SK. The *in vivo* activation of animal plasminogen by a mixture of SK and human plasma could depend on not only the total dose but also on such factors as the time of administration after mixing, the amount of SK and human plasminogen used, and the deterioration characteristics of the SK-plasminogen mixture.

It should be evident that the fibrinolytic activity appearing in an animal after administration of a mixture of SK and human plasminogen would reflect not only the human plasmin administered but also the activated plasminogen of the animal.

Summary. The ability of a mixture of SK and human plasminogen to activate bovine plasminogen was shown to be dependent upon the concentrations of both components. The 'activator' which was formed, may deteriorate on incubation even though the SK and human plasminogen when incubated separately were stable. The decrease was partially or completely restored by the addition of SK. The rate of disappearance of the 'activator' was affected by the plasminogen preparation and by the initial SK concentration. The inactivation occurred at physiological pH as well as at pH 6.4. The *in vitro* and *in vivo* significance of the data were discussed.

We are grateful to Mr. John Poletto for the LEE preparation, Mr. Merle Querry for some plasminogen fractions, to Mrs. Emma Zilko for carrying out certain assays and to Drs. Edward DeRenzo and Samuel Gordon for their helpful suggestions in preparing this manuscript.

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Received June 29, 1956. P.S.E.B.M., 1956, v93.

Studies on Phenylketonuria. V. Observations on a Newborn Infant with Phenylketonuria.* (22775)

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Phenylketonuria, an inherited biochemical abnormality in the oxidation of phenylalanine to tyrosine, is usually associated with mental deficiency(1). Some of the pathological symptoms may be due to the presence of some toxic substance formed as a consequence of the greatly increased amount of phenylalanine which accumulates in patients(2-5). It was important to determine whether newborn infants with phenylketonuria are biochemically normal, since the rate of development of biochemical abnormalities might relate both to the age of onset of symptoms and to the severity of the mental defect observed in later life. There have been indications(5,6) that the urine of newborn infants later found to have phenylketonuria gives a negative reaction for phenylpyruvic acid when tested with ferric chloride. This test is unreliable, however, in establishing the absence of phenylpyruvic acid from dilute samples of urine such as those excreted by small infants, and, in order to determine the degree of biochemical abnormality, it is necessary to use other tests. These are the 2,4-dinitrophenylhydrazine test for phenylpyruvic acid, paper chromatography of an extract of urine in order to detect phenylpyruvic, *o*-hydroxyphenylacetic(7), *p*-hydroxyphenyllactic and indolelactic acids, and determinations of serum levels of phenylalanine. Arrangements were made to obtain blood and urine from infants born to parents who had previously had a child with phenylketonuria. The third infant studied in this manner was phenylketonuric.

Methods. Cord blood was collected immediately after parturition; blood from the newborn infant was collected within 3 hours of birth and at intervals throughout the first month. Serum phenylalanine was determined by a modification(4) of the method of Kapeller-Adler. Urine was collected as frequently as was feasible throughout the first month; unfortunately, the infant observed was a girl, it was impractical to hospitalize her, and urine samples were small, though sufficient for these studies. For qualitative test, the dinitrophenylhydrazine reagent was added to an equal volume of urine; a positive test is shown by the appearance of a yellow turbidity at concentrations of phenylpyruvic acid as low as 2 mg %. This test has proved to be far more reliable than the ferric chloride test for phenylpyruvic acid. Urine was extracted by the procedure described earlier(8); relatively larger volumes of solvents than usual were required because of the small amount of urine available, but the final ethyl acetate solution of the organic acids was conc. in a stream of air and the final volume was adjusted by the addition of ethyl acetate. Small amounts of phenylpyruvic acid may be detected on 2-dimensional chromatograms by the development of a brown color with ammoniacal $AgNO_3$(9); larger amounts are evident as a diffuse pink area on chromatograms which have been sprayed with diazotized sulfanilic acid(12) (phenylpyruvic acid, R_F 0.58 in isopropyl alcohol-aqu. $NH_3 - H_2O = 8:1:1$ (Ipr – NH_3) and 0.66 in benzene-propionic acid-water = 2:2:1 (organic phase) (Bz-prop.). *o*-Hydroxyphenylacetic acid was estimated as described previously(8), indolelactic acid by spraying 2-dimensional chromatograms with *p*-dimethylaminobenzaldehyde (purple color, R_F 0.48, Ipr-NH_3, and 0.38, Bz-prop.) and *p*-hydroxyphenyllactic acid with diazotized sulfanilic acid.

Clinical summary. Baby M was born at

* This work was supported by research grants from the N.I.H., U. S. Public Health Service.

term of an uneventful pregnancy; birth weight, 5 lb. 12½ oz. She was completely normal in all respects when followed in the hospital during the newborn period and when examined at the age of one month. She was breast fed and received adequate nourishment. An electroencephalogram, made at the age of 33 days showed evidences of abnormalities. These included isolated spikes and some spike and slow wave complexes of irregular contour, observed in both the waking and sleep records. The record was suggestive of a diffuse cortical disturbance, which appeared to be more marked over the right hemisphere. In order to avoid possible damage to the child, the studies were not continued after phenylpyruvic acid appeared in her urine. She was brought into the hospital at the age of 33 days, and at the age of 40 days she was started on a phenylalanine-deficient regimen.†

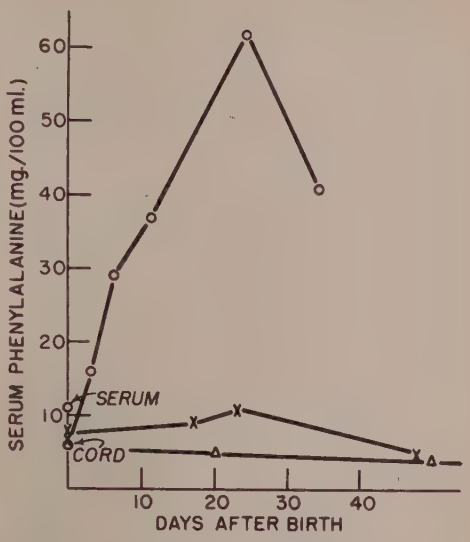

FIG. 1. Serum phenylalanine levels in newborn infant with phenylketonuria. ○, Baby M. Serum sample drawn just after birth was badly hemolyzed. A higher value was obtained than with cord blood, probably because of interference with the normal chromogen. ×, Baby U; △, Baby L.

Results. The results of the serum phenylalanine determination are shown in Fig. 1; for comparison, values are included from the 2 infants studied previously who proved to be normal. The slow gradual rise of the phenylalanine level in Baby M is in contrast to the fairly rapid (1 day) rise to high levels in older patients with phenylketonuria who were maintained at normal blood phenylalanine levels by a phenylalanine-restricted regimen and then given a natural diet(4). The chromatographic study of the excretion of the minor abnormal metabolites, *o*-hydroxyphenylacetic, indolelactic, and *p*-hydroxyphenyllactic acids showed that, at 5 days of age and with a phenylalanine level of 24 mg %, the infant excreted *none* of the abnormal metabolites always observed in older phenylketonurics. The next urine sample (16 days of age, serum phenylalanine, 47 mg %), contained greatly increased amounts of the above acids, although the rate of excretion of *o*-hydroxyphenylacetic acid was only one-fifth of that observed in older patients with a comparable serum phenylalanine level. The rate of excretion of *o*-hydroxyphenylacetic acid then remained at about one-fifth that observed in older patients.

The most striking finding was that the baby did not excrete a detectable amount of phenylpyruvic acid until it was 34 days old. At this time the urine contained no more than 0.2 mg phenylpyruvic acid per mg creatinine and the daily excretion could have been no more than 7 mg. The excretion of phenylpyruvic acid by most patients is 1.5-3.0 mg/mg creatinine. Phenylpyruvic acid also was clearly detectable for the first time on a 2-dimensional chromatogram of the organic acids extracted from this sample of urine.

Discussion. The possible absence of phenylpyruvic acid from the urine of phenylketonuric infants before the age of one month calls for caution in arriving at a negative diagnosis based on testing during the newborn period the urine of siblings of older children with phenylketonuria. A determination of serum phenylalanine would provide a more reliable test. Although the present report is

† The diet was similar to that reported previously (4), with the exception that a phenylalanine-deficient casein hydrolysate, supplemented appropriately with amino acids, was used in place of the amino acid mixture.

of a single case, the delay in the development of typical biochemical abnormalities is consistent with previous observations that some phenylketonuric children may develop normally for a few months before developing pathological symptoms(4,5). It might be expected that further observation on other newborns with phenylketonuria will reveal considerable differences in the time required for phenylpyruvic acid to appear in their urine.

The delayed appearance of phenylpyruvic acid might be explained most simply by the hypothesis that phenylalanine transaminase is not present in newborn infants with phenylketonuria, and that the production of phenylpyruvic acid and the accompanying fall in serum phenylalanine level indicate the maturation of this enzyme system. A similar development of the tyrosine oxidase system has been shown to occur around the time of birth (10).

Summary. Biochemical studies have been performed on a phenylketonuric infant from birth to the age of 34 days. Cord blood and newborn infant blood contained normal levels of phenylalanine, the serum phenylalanine rose to 62 mg % at 24 days, and decreased to 41% at 34 days. Phenylpyruvic acid was not excreted in detectable amounts until 34 days. *o*-Hydroxyphenylacetic acid was not present in excess at 5 days, but from 16 to 34 days was excreted at about one-fifth the rate usually observed in phenylketonuria. The minor abnormal metabolites indolelactic and *p*-hydroxyphenyllactic acids were not present at 5 days, but were present at 16 days.

We gratefully acknowledge the help of Dr. Stanley W. Wright, Dept. of Pediatrics, University of California Medical Center, Los Angeles, and of Drs. James F. Bosma and Niels L. Low, Dept. of Pediatrics, Univ. of Utah College of Medicine, who obtained materials from the 2 normal infants reported in this study. Eleanor Bethsold carried out the analytical work. We are indebted to Drs. F. A. Horner and C. W. Streamer, Dept. of Pediatrics, Univ. of Colorado Medical Center for their interest and aid in furthering this work. Dr. Lynwood M. Hopple, Children's Hospital, Denver, Colorado, evaluated the E E G. Merck Sharp and Dohme kindly supplied the phenylalanine-deficient basal mix which was given the infant.

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Received July 6, 1956. P.S.E.B.M., 1956, v93.

Glyoxal and Related Compounds as Potential Blood Sterilizing Agents. (22776)

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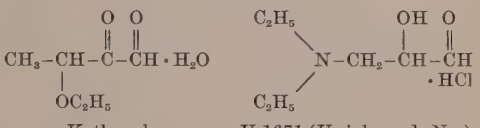
A major problem in the use of human blood and plasma for transfusions is the danger of transmitting the virus of serum hepatitis. No completely satisfactory method is available for inactivation of the virus. Ultraviolet irradiation(1), β-propiolactone (BPL)(2) and a combination of these(3,4) have been studied. Since preliminary studies in our laboratory indicated that glyoxal and many related compounds were potent virucidal agents, experiments were undertaken to determine the potential value of these compounds as blood sterilizing agents by comparing their *in vitro* virucidal activity and their toxicities with other known virucidal agents. Two of these compounds, glyoxal and methyl glyoxal, were previously tested but apparently discarded because when "added to human blood plasma, they caused an increase in the electrophoretic mobilities at pH 8.6 of the plasma proteins."

Materials and methods. Viruses: (a) influenza A, PR-8 strain, supplied by Dr. Thomas Francis, Jr., University of Michigan. It was an egg-adapted strain, lethal for 10-day embryos within 60-80 hrs; it reached a titer in 10-day eggs of about 10^9 LD_{50} per 0.1 ml when injected into the allantoic cavity. (b) Newcastle disease virus, NJ-KD strain, secured from Dr. E. S. Pomeroy, University of Minnesota. It was an egg-adapted strain, lethal for 10-day embryos within 50-70 hrs; it reached a titer in 10-day eggs of about 10^8 LD_{50} per 0.1 ml when injected into the allantoic cavity. (c) Mouse hepatitis virus, received from Dr. J. B. Nelson(5). A stock was prepared by injecting 0.1 ml volumes of a 10^{-2} dilution of the virus by the intraperitoneal route into a group of Princeton weanling mice (Millerton Research Farm, Millerton, N. Y.). Fifty hours after infection, the livers of the mice were harvested and a 10% suspension in beef infusion broth was prepared. This material had a titer of approximately 10^7 LD_{50} per 0.1 ml in Princeton weanling mice when injected intraperitoneally.

Chemicals:* (a) *Glyoxal*—A specially purified aqueous solution was obtained from Carbide and Carbon, Inc. The colorless solution was passed through Amberlite IR-45 resin to remove any acid present. The glyoxal content of the resulting solution was 36.8% by weight. (b) Pyruvaldehyde (methyl glyoxal)—This was obtained from the same source and purified in the same way as the glyoxal. The methyl glyoxal content of the aqueous solution was 33.4% by weight. (c) Formaldehyde—A fresh commercial sample (37% aqueous solution) was filtered through paper and used directly. (d) β-propiolactone—A redistilled commercial sample (B. F. Goodrich Co.) was used. To minimize hydrolysis, the BPL dilutions were made immediately before using. Water was used as the solvent except when the lysis of erythrocytes was being studied; then saline was used. (e) Kethoxal (β-ethoxy-α-ketobutyraldehyde hydrate); U-1671 (β-diethylaminolactaldehyde hydrochloride); and Kethoxal bisulfite—A description of the synthesis of these compounds will be published elsewhere. The structures of Kethoxal and U-1671 are shown below:



Kethoxal

U-1671 (Upjohn code No.)

Test for virucidal activity. One of 3 media was used: (a) A mixture of equal volumes of beef infusion broth and physiological saline, (b) whole blood, (c) plasma. Virus was incubated for 2½ hrs at 37°C with equimolar quantities of the various test chemicals. The

* The chemical synthesis and purification procedures were conducted by Dr. R. H. Levin and his associates in the Chemistry Department of The Upjohn Co.

amount of infective virus remaining in each sample after incubation was determined by making serial 10-fold dilutions in 1:1 beef infusion broth:saline and titrating in groups of 10 eggs or mice, depending on the virus used. Deaths were recorded for a period of 10 days, and the titers were calculated by the method of Reed and Muench(6).

Test for Lysis of Erythrocytes. Equimolar quantities of the test chemicals were mixed with 10 ml of 1% human red blood cells suspended in physiological saline. Five tubes for each chemical and 5 control tubes were prepared. The tubes were continuously shaken on a mechanical shaker at 25°C. The amount of lysis was determined at intervals by centrifuging one tube from each set for 5 min at 2000 rpm in a Model V International Centrifuge and determining the optical density of the supernatant at a wavelength of 590 mμ in a Beckman Model B Spectrophotometer. A standard curve was previously drawn in which optical density was plotted against known concentrations (0.1-1.0%) of red blood cells which were completely lysed by placing them in distilled water for 10 min. The % lysis of the test samples was determined by referring optical density measurements to this standard curve. A control tube which had been shaken for the same length of time served as the blank.

Electrophoresis studies. Equimolar amounts of the test chemicals were added to human plasma. After incubation under varying conditions, each sample was diluted with an equal volume of veronal buffer, pH 8.6 and ionic strength 0.1 and dialysed against 250 ml of the same buffer for 24 hrs. The samples were then diluted to approximately 1.5% total protein concentration and dialysed for another 24 hr against 2 L of fresh veronal buffer. The dialysed samples were then subjected to electrophoresis for a period of 3 hr.†

Results. Comparative in vitro virucidal activity of various chemicals. Comparative virucidal activities of several of the test chemicals were determined (Table I). Kethoxal was a more potent virucidal agent in broth, plasma or whole blood medium than were equimolar amounts of BPL, formaldehyde or U-1671. Experiment 3 (Table I) further indicated that Kethoxal and glyoxal were more potent virucidal agents for PR-8 virus in whole blood medium than were any of the other chemicals tested; that a molar concentration of approximately 6.75×10^{-3}

† The electrophoresis determinations were conducted by G. C. Colovos of The Upjohn Company, Physics Department.

TABLE I. Comparative Virucidal Activities of Various Chemicals vs. Influenza A (PR-8) and Newcastle Disease (NJ-KD) Viruses.

Equimolar quantities of the test chemicals, each in a vol of 0.1 ml, were incubated with 0.1 ml of 10-fold diluted (Exp. 1) or non-diluted (Exp. 2 and 3) infective allantoic fluid in 4.8 ml of the indicated medium. Each sample was incubated for 2½ hr at 37°C, and the amount of residual infective virus was then determined by making serial 10-fold dilutions and titrating in groups of 10 eggs. Each dilution was inj. intra-allantoically at a level of 0.5 ml/egg. Results are expressed as the $\log_{10}$ titer of 1 LD_{50}.

Exp. No.*	Chemical	Molar conc.	Final titer
1 a	BPL	1.35×10^{-4}	8.7
	Formaldehyde	"	8.9
	Kethoxal	"	6.5
	U-1671	"	8.4
	Control	—	9.0
1 b	BPL	1.35×10^{-4}	6.9
	Formaldehyde	"	7.4
	Kethoxal	"	5.6
	U-1671	"	7.0
	Control	—	7.5
2	BPL	1.35×10^{-4}	8.7
	Formaldehyde	"	8.9
	Kethoxal	"	7.5
	U-1671	"	8.5
	Control	—	9.1
3 a	Kethoxal	2.70×10^{-3}	6.7
	"	4.05×10^{-3}	5.1
	"	5.40×10^{-3}	2.8
	"	6.75×10^{-3}	<2.0
	BPL	"	6.0
	Formaldehyde	"	5.3
	U-1671	"	8.1
	Kethoxal bisulfite	"	6.2
	Glyoxal	"	<2.0
	Methyl glyoxal	"	7.4
	Control	—	8.1
3 b	Kethoxal	2.70×10^{-3}	5.1
	Glyoxal	"	4.2
	Control	—	6.4

* The medium used in Exp. 1 was 50% beef broth, in Exp. 2 was chicken plasma, and in Exp. 3 was chicken blood. The PR-8 virus was used in Exp. 1 a, 2 and 3 a; NJ-KD was used in Exp. 1 b and 3 b. The initial titer of the viruses in each exp. was as follows: 1 a = 9.4; 1 b = 7.9; 2 = 9.2; 3 a = 9.3; 3 b = 7.6.

TABLE II. Comparative Virucidal Activities vs. Mouse Hepatitis Virus.

Equimolar quantities of the test chemicals, each in a vol of 0.1 ml, were incubated with 0.5 ml of a 10% liver suspension of mouse hepatitis virus in 4.4 ml of whole chicken blood. Each sample was incubated for 2½ hr at 37°C, and the amount of residual infective virus was then determined by making serial 10-fold dilutions and titrating in Princeton weanling mice. Each dilution was inj. intraper. at a level of 0.1 ml/mouse. Deaths were recorded for a period of 10 days. Results are expressed as the reciprocal of the $\log_{10}$ titer of 1 LD_{50}.

Exp. No.	Chemical	Molar conc.	Final titer
1	Kethoxal	6.75×10^{-3}	2.1
	BPL	"	3.4
	Glyoxal	"	<2.0
	Control	—	6.8
2	Kethoxal	3.38×10^{-3}	4.0
	BPL	"	6.1
	Glyoxal	"	3.6
	Control	—	7.0

(1000 mg/L) of Kethoxal was required to inactivate all PR-8 virus in whole blood under the stated incubation conditions; and that glyoxal was slightly more potent than Kethoxal against NJ-KD virus in whole blood. The comparative virucidal activities of Kethoxal, BPL and glyoxal against the virus of mouse hepatitis were studied. This virus was chosen because of its possible closer relationship to the virus of serum hepatitis. The results (Table II) indicated that Kethoxal and glyoxal were more active than BPL against the virus of mouse hepatitis.

Comparative effect on lysis of erythrocytes. An experiment was designed to test the comparative effects of Kethoxal, BPL and glyoxal on a 1% suspension of human red blood cells. All 3 chemicals were used at a molar concentration of 6.75×10^{-3}. The results (Table III) indicated that BPL lysed the red cells more rapidly than did Kethoxal or glyoxal. When whole blood was used, the results were different. Equimolar amounts of the chemicals were mixed with whole human blood and stored for 3 weeks at 4°C, similar to storage conditions used in hospital blood banks. Under these conditions, it was found that a molar concentration of 4.05×10^{-2} (6000 mg/L) of Kethoxal caused 2.3% lysis of the erythrocytes of whole blood, an equimolar amount of BPL caused 2.0% lysis, and an equimolar amount of glyoxal caused 1.4% lysis. A control sample of human blood stored for the same period of time was found to have undergone 1.1% lysis of the red cells, indicating that the net lytic effect of the glyoxal (0.3%) was very low. Spectrophotometer readings on the supernatants from these whole blood samples were made at a wavelength of 490 mμ. The lower wavelength was used with the whole blood samples because at 490 mμ there was less interference from the yellow color apparently formed by reaction of the carbonyl compounds with blood proteins. No attempt was made in these experiments to correct the values for possible formation of methemoglobin.

TABLE III. Comparative Effect of Equimolar Amounts of Kethoxal, β-Propiolactone and Glyoxal on Lysis of a 1% Suspension of Human Red Blood Cells.

Kethoxal was used at a molar conc. of 6.75×10^{-3} (1000 mg/L). The figures in the table express % of lysis.

Time after mixing (hr)	Kethoxal	β-Propiolactone	Glyoxal
1	0	41	0
2	0	57	0
4	0	85	0
6	23	92	0
7	31	95	0

Comparative effect on plasma proteins. In order to determine the effect of Kethoxal on plasma proteins, a fresh sample of human plasma was obtained and treated with Kethoxal at a molar concentration of 6.75×10^{-3} (1000 mg/L); this was the minimum concentration previously found to destroy all detectable Newcastle and influenza viruses in artificially infected whole blood. A second portion of the plasma was treated with an equimolar amount of glyoxal, a third portion with an equimolar amount of BPL, and a fourth portion served as a control. Each sample was incubated for 93 hr at room temperature, then for an additional 48-72 hr at 4°C. In a similar second experiment, one group of samples received the 3 chemicals at a molar concentration of 6.75×10^{-3}, as above; another group of plasma samples received double this amount of each chemical.

This group of 8 samples, 6 treated and 2 controls, was incubated for 21 days at 4°C, then subjected to electrophoresis. It was found that the presence of the various chemicals under the stated incubation conditions caused no significant changes in the electrophoretic pattern of the human plasma.

Discussion. The results of these experiments indicate that glyoxal and Kethoxal may have value as virucidal agents. Both chemicals were more potent in destroying the infectivity of influenza A, Newcastle disease and mouse hepatitis viruses than was an equimolar amount of BPL. Glyoxal appeared to be less lytic than BPL or Kethoxal, and none of the chemicals caused significant changes in human plasma proteins at concentrations which were effective in sterilizing artificially-infected blood. These results suggest that glyoxal and Kethoxal hold some promise as blood-sterilizing agents. Glyoxal gave results as good or better than Kethoxal. Since thorough studies have not been made on the toxicity of glyoxal for mammalian hosts(7,8), further work is required before it can be applied to the large-scale sterilization of blood and plasma. The marked superiority of glyoxal over formaldehyde as a virus inactivating agent suggests also the possible application of glyoxal in the preparation of "killed" vaccines or as a general viral disinfectant.

Summary. Both glyoxal and β-ethoxy-α-ketobutyraldehyde (Kethoxal) were more potent virucidal agents than β-propiolactone (BPL). Glyoxal was less lytic for human erythrocytes than was Kethoxal or BPL. None of the chemicals showed significant toxicity for plasma proteins as measured by electrophoresis. These experiments indicate that glyoxal may be of potential value in the sterilization of human blood and plasma.

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Received July 16, 1956. P.S.E.B.M., 1956, v93.

Increased Erythropoietic Stimulant in Plasma of Pregnant Rats.* (22777)

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The existence of an erythropoietic substance in the blood stream was first suggested by Carnot and Deflandre(1) who showed that plasma from rabbits which had been bled repeatedly when injected into other rabbits resulted in hyperplastic bone marrow and increased reticulocyte and erythrocyte counts. Several investigators have since(2-19) confirmed the presence of erythropoietic activity in plasma of animals which had been bled. Plasma from animals exposed to low oxygen tensions has similarly been found to stimulate erythropoiesis in recipients (6b,9,20-22, 24b). Human umbilical cord blood, also fetal sheep and dog blood, have been found to increase red blood cell count in recipient animals(22-26). As these reports of an erythropoietic substance in blood were based either

* Supported by grants from the United States Public Health Service, A-366 and A-661.

† The authors wish to acknowledge the continued assistance and cooperation of Dr. John H. Lawrence and staff of the Donner Laboratory in studies on erythropoiesis.

on reticulocyte counts, red blood cell counts, or hemoglobin determinations, and as these methods are subject to error introduced by changes in plasma volume, it is not surprising that other investigators have been unable to confirm the presence of an erythropoietic substance in blood(27-29).

It was the object of this study to reinvestigate the existence of an erythropoietic stimulant in the blood stream of the rat, using a more satisfactory method for determination of increased numbers of red cells in the circulation, namely, the total circulating red cell volume. Changes which may exist under differing physiological conditions, such as pregnancy and fetal life, would then be on a more sound basis. This paper compares the levels of erythropoietic factor in plasma of pregnant and normal female rats.

Material and methods. The test animals for measuring red cell volume were female rats of the Long-Evans strain, hypophysectomized on the 26th to the 28th day of life and maintained for 45 to 50 days to allow stabilization of the hematological values at the low level characteristic of hypophysectomized rats. The anemia which develops within this period in such animals is severe, the red cell volume being 50% below normal. This anemia is known to be promptly repaired after administration of erythropoietic stimulants such as cobalt, plasma factor and pituitary extracts(15,30-32). The plasma from pregnant rats was newly prepared for each day's injection, as follows: Between the 15th and 18th days of pregnancy the donors were exsanguinated by withdrawal of blood from the aorta into a heparinized syringe. The blood was transferred at once to centrifuge tubes containing a small amount of mineral oil to exclude air, and was centrifuged for 12 minutes at 2,000 rpm. The plasma was drawn into a syringe and immediately injected intraperitoneally. This procedure was repeated daily for 14 days. Groups consisting of 6 to 8 hypophysectomized rats received daily doses of 2 or 4.8 cc. Other groups received 2 or 4.8 cc of plasma similarly prepared from normal adult female rats of the same age as the pregnant donors. At the termination of the ex-

TABLE I. Increased Hematological Values of Hypophysectomized ($\bar{H}$) Rats Injected with Plasma from Normal (N) or Pregnant Rats.

Type of plasma	Daily dose 14 d, ml	No. rats	Body wt, g*	Hemoglobin, g		Blood vol per 100 g body wt, ml	Plasma vol per 100 g body wt, ml	Hematocrit, %	Red cell vol, ml	
				Per 100 ml	Total	Per 100 g body wt			Total	Per 100 g body wt, ml
Pregnant	2	26	82 ± 1	9.3 ± .2	.42 ± .02	.51 ± .01	5.52 ± .10	3.85 ± .20	30.4 ± .2	1.38 ± .12	1.68 ± .05
	4.8	7	84 ± 1	10.3 ± .1	.56 ± .01	.67 ± .02	6.44 ± .15	4.45 ± .10	30.8 ± .1	1.67 ± .09	1.99 ± .03
Normal	2	22	85 ± 1	9.2 ± .2	.40 ± .01	.47 ± .02	5.18 ± .18	3.73 ± .08	28.5 ± .1	1.23 ± .11	1.45 ± .02
	4.8	6	81 ± 1	10.0 ± .2	.47 ± .01	.58 ± .01	5.82 ± .15	4.04 ± .10	30.5 ± .1	1.44 ± .11	1.78 ± .02
Controls											
$\bar{H}$		19	76 ± 1	9.3 ± .2	.34 ± .02	.45 ± .01	4.80 ± .07	3.41 ± .09	28.9 ± .1	1.06 ± .07	1.39 ± .04
N		29	198 ± 3	13.0 ± .2	1.32 ± .02	.67 ± .02	5.13 ± .08	2.88 ± .08	43.9 ± .5	4.46 ± .09	2.25 ± .03

* Staud. error = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

periment control hematological values were determined in uninjected hypophysectomized and normal rats of the same age as the experimental groups. Red cell volumes of all rats were determined by the Fe^{59} tagged cell method(31). The result obtained from injection of the lower dose level (2 cc of plasma) was confirmed 3 times. All hypophysectomized rats were fed a standard laboratory diet (Diet XIV) consisting of: wheat 68.5%, casein 5%, fish meal 10%, alfalfa leaf meal 10%, fish oil 5%, NaCl 1.5%, KI added (analysis 1 μg iodine per g diet). Each afternoon a dish of liquid diet was given consisting of Diet I (modified McCollum Diet I): wheat 67.5%, casein 15%, skim milk powder 7.5%, fish oil 1%, NaCl 0.75%, $CaCO_3$ 1.5%, KI added (analysis 1 μg iodine per g diet). Lettuce was fed twice weekly. Pregnant and lactating rats were maintained on Diet I.

Results. The results are presented in Table I. The hypophysectomized rats injected with the daily dose of 2 cc of plasma from pregnant rats showed a significant increase in total circulating red cell volume (21%), also a significant increase in red cell volume per 100 g of body weight ($P = <.001$). The increase in circulating red cell volume was accompanied by an increase in total circulating hemoglobin of 14%, and of 13% in hemoglobin per 100 g of body weight ($P = <0.01$). Injection of the larger dose of plasma from pregnant rats (4.8 cc) increased total circulating hemoglobin by 51%, the hemoglobin per 100 g of body weight by 40%, circulating red cell volume and red cell volume per 100 g body weight by 43% ($P = <.001$). No increase in hematological values occurred in hypophysectomized rats injected with the 2 cc dose of plasma from normal rats. At the higher daily dose of normal plasma (4.8 cc) which was equivalent to, or greater than, the recipient's total blood volume, an increase of 23% in red cell volume per 100 g body weight ($P = <.001$), and of 21% in hemoglobin per 100 g body weight resulted ($P = <.001$). The stimulation to erythropoiesis, when normal plasma was used, was definitely less than that observed from injection of plasma from pregnant rats ($P = <.001$).

The hematocrit and hemoglobin/100 ml were not increased in any injected hypophysectomized rats as there was a concomitant increase in plasma volume and in total blood volume. Increase in plasma volume was more pronounced in those injected with plasma from pregnant rats, ranging from 13% ($P = <.001$) at the lower dose level to 34% ($P = <.001$) at the higher level. The increase in plasma volume in rats injected with normal serum was only 8% ($P = <.001$) at the lower dose level, increasing to 19% ($P = <.001$) at the higher level. The increased plasma volume probably is attributable to the growth promoting substances (or growth hormone) which has been shown to be present when this amount of plasma from normal or pregnant rats was given. Pituitary growth hormone has been shown to increase plasma volume(34).

Increase in red cell volume and hemoglobin in hypophysectomized rats injected with plasma from normal rats is further evidence that a circulating erythropoietic factor is normally present in the blood stream, and the higher values in rats injected with plasma from pregnant rats indicates that increased amounts of erythropoietic substance circulate during pregnancy. Increased production during pregnancy of this factor, or factors, may be responsible for the adult level of the red cell volume which characterizes the rat at birth; withdrawal of this stimulus at birth may account for the physiological anemia developing in the neonatal period(33). If this is true, then plasma from pregnant rats should prevent the neonatal anemia.

To test this concept newborn rats were injected during the postnatal period with plasma from pregnant rats and the results were compared with those from injection of normal rat plasma. Experimental groups were so constituted that each group consisted of 6 male rats, each from a different litter. Two of the 6 rats received normal rat plasma, 2 received pregnant rat plasma, and 2 were uninjected controls. (The newborn rats were identified by clipping toes, or by their color and coat pattern.) Care was taken to maintain the mothers on a diet satisfactory for lactation. The injections were started the 4th

TABLE II. Prevention of Neonatal Anemia by Injections of Pregnant Rat Plasma.

Type of plasma inj.	No. rats	Body wt, g*	Hemoglobin, g		Blood vol, ml	Plasma vol, ml	Hematocrit, %	Red cell vol, ml	
			Per 100 ml	Total	Per 100 g body wt			Total	Per 100 g body wt
Pregnant	40	45.0 ± .5	6.8 ± .1	.21 ± .01	.46 ± .01	6.78 ± .10	4.78 ± .08	29.5 ± .3	.90 ± .03	2.00 ± .03
Normal	29	45.2 ± 1.0	6.3 ± .1	.19 ± .01	.41 ± .01	6.52 ± .09	4.89 ± .10	27.6 ± .7	.83 ± .01	1.83 ± .04
No inj.	45	44.1 ± .5	6.7 ± .2	.18 ± .01	.41 ± .01	6.25 ± .10	4.60 ± .07	26.4 ± .3	.73 ± .01	1.66 ± .02

* Stand. error = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

day of life and were continued daily for 14 consecutive days or until the rats were 18 days of age at which time the neonatal anemia is known to be most severe(33). The suckling rats were removed from cages only for the short periods required for injection. After injection each group of 6 suckling rats was placed with a different lactating female in order to equalize the milk supply. With proper care the young were not rejected because of this procedure.

The plasma was prepared by the procedure described previously. The dose given was 1 cc per day for the first 5 days, and 2 cc per day for the remainder of the experimental period.

The combined results of 4 repetitions of this experiment are presented in Table II. A statistically significant ($P = <.001$) increase (22%) occurred in the red cell volume of the newborn rats injected with plasma from pregnant rats. A smaller increase, 10% ($P = <.05$), occurred when the newborn rats received plasma from normal female rats. A significant increase also occurred in other hematological values. The anemia of the newborn rat was ameliorated, though not completely prevented, by injection of plasma from pregnant rats; the values were 10% below normal adult levels but were 22% above typical postnatal levels. From the results obtained in newborn rats, as well as in hypophysectomized rats, it may be concluded that the plasma from pregnant rats has an erythropoietic potency greater than that of the plasma from normal adult female rats. These results therefore support the assumption that an erythropoietic principle in pregnant rat serum plays a role in fetal red cell production. The site of the production of this erythropoietic factor is under investigation.

Summary. 1. Plasma of normal adult female rats contains a factor capable of stimulating erythropoiesis in hypophysectomized and newborn rats, as judged by increased circulating red cell volume and hemoglobin. 2. This humoral erythropoietic factor is markedly increased during pregnancy. 3. Injections of plasma from non-pregnant and pregnant rats resulted in an increase in plasma

and blood volumes of hypophysectomized and newborn recipients.

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Received July 19, 1956. P.S.E.B.M., 1956, v93.

Production of Anti-Human PTC and Anti-Human Proconvertin in Rabbits.* (22778)

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The appearance of circulating anticoagulants has been described in both hemophilia A(1-12) and hemophilia B (PTC deficiency, Christmas Disease)(13,14). Presumably, in both diseases, the inhibitors have arisen as antibodies against "foreign" proteins, either the AHF or the PTC of therapeutically infused normal plasma. In each disease it has been possible to demonstrate that the inhibitor is specific to the factor missing in the particular patient(15). The preparation, in animals, of similar specific antibodies to the various human clotting factors could prove of great value in the study of blood coagulation and, if precipitin reactions occur, in assays of the various factors. Pinniger and Prunty (16) and Hardisty and Pinniger(17) describe the preparation, in rabbits, of an anti-human fibrinogen giving positive precipitin reactions to human plasma and fibrinogen but negative to serum and the plasma of an afibrinogenemic patient. Recently Richards and Spaet (18) have, by immunization of rabbits with human AHF, prepared a rabbit serum highly inhibitory to human AHF, and giving positive precipitin tests to the antigen. However, they do not describe precipitin tests with normal plasma, serum, or hemophilic plasma.

These studies were undertaken in an attempt to prepare rabbit antisera with specific anti-PTC activity. Rabbits injected with our "PTC" preparations formed inhibitors to both PTC and proconvertin. Attempts were made to separate and further purify the antiserum by adsorption with human plasmas from patients congenitally deficient in PTC or proconvertin.

Methods. Preparation of serum PTC. A number of different PTC preparations were made, often with slight modifications of the following: Outdated citrated blood bank plasma was dialysed 48 to 72 hours against 0.01 M sodium oxalate in 0.85% NaCl, recalcified with 1/50 volume 1 M $CaCl_2$, allowed to stand overnight (4°C) and the clot removed. Barium sulfate, 10 mg/ml, was stirred for 10 min. with the serum, centrifuged, washed twice with 0.85% NaCl, twice with distilled water and eluted twice with 1/10 the plasma volume of 0.2 M sodium citrate. The eluates were combined, dialysed 24 hours against water and lyophilized to 1/20 the original plasma volume. The specific resistance was adjusted to that of 0.85% NaCl and the pH to 7.1. Tests on these "PTC" fractions revealed that they contained 5 to 10 times the plasma concentration of PTC, some proconvertin, little prothrombin and no detectable thrombin, AHF, proaccelerin, Hageman factor or fibrinogen. Initial attempts at immunization involved intravenous injections in 3 rabbits, 2 of which convulsed and died within seconds after the fifth injection and showed adherent clots in the ventricles. The remaining 8 rabbits were immunized with intramuscular injections: 1 ml 3 times a week for the first week, 2 ml 3 times a week for the second week, a 10 day rest, followed by a 5 ml injection. Blood was obtained by cardiac puncture 12 and 24 days later. Initial tests, employing rabbit serum, frequently caused clotting when plasma was present in the system. Therefore, in most instances, whole or $BaSO_4$ absorbed oxalated rabbit plasma was used. *Plasma Fractions.* AHF: 0.5 ml rabbit plasma was mixed with 0.1 ml 95% alcohol, chilled 1 hour, the precipitate collected and dissolved in 0.5 ml 0.85% NaCl. *PTC:* 1.5 ml rabbit plasma was mixed with 150 mg $BaSO_4$, centrifuged, the $BaSO_4$ washed once with distilled water,

* This study was supported by research grants from N.I.H., U.S. Public Health Service, and Pittsburgh Chapter of Hemophilia Foundation, inc.

† Postdoctorate Fellow, Life Insurance Medical Research Fund.

drained and eluted with 0.5 ml 0.2 M sodium citrate. This eluate was diluted 1-10 before testing, and contained prothrombin and proconvertin as well as PTC. *Clotting tests.* Most of the methods have been described(19, 20). Hageman factor was measured in a manner similar to AHF, but employing as a substrate plasma from a patient with Hageman factor deficiency(21). This patient was seen through the courtesy of Dr. Oscar Ratnoff. Titers for AHF, PTC, Hageman factor, prothrombin, proconvertin and proaccelerin were carried out on 1-10 dilutions of plasma. Tests for inhibitors to these factors were carried out by incubating 1-5 diluted normal human plasma with equal volumes of normal or injected $BaSO_4$ adsorbed rabbit plasma or saline for 30 minutes and then measuring the residual activity in each test system. When this activity was less than that of the saline-incubated human plasma, inhibition had occurred. Complete inhibition was recorded as 4+. Antifibrinogen was measured by incubating equal volumes of whole human plasma and whole rabbit plasma and, after 30 minutes, measuring fibrinogen concentration of the mixtures turbidometrically(2). This concentration could then be compared to that calculated for the mixtures, the concentration of each component having been measured separately. Anti-thrombin activity was estimated by comparing the clotting times of normal and injected rabbit plasma after addition of human thrombin. Anti-thromboplastin was measured by comparing clotting times of normal and injected rabbit plasmas, mixed with human brain suspension for 30 minutes and recalcified. Anti-human plasma thromboplastin was measured by allowing a normal human thromboplastin generation (modified from(22)) mixture (0.2 ml $BaSO_4$ plasma (1-5) + 0.2 ml serum (1-10) + 0.2 ml platelet suspension + 0.2 ml 0.025 M $CaCl_2$) to incubate 6 minutes, removing 0.1 ml samples, incubating these with 0.1 ml of control or injected rabbit $BaSO_4$ adsorbed plasma or with 0.85% NaCl for 5 minutes and recording the clotting times on addition of 0.1 ml human citrated plasma and 0.1 ml 0.025 M $CaCl_2$.

Results. Blood coagulation studies in the rabbits. (Table I, Fig. 1). Clotting times, prothrombin consumptions and platelet counts carried out on one rabbit from the injected group and one from the control group did not show any significant differences. The 8 injected rabbit plasmas all showed anti-human PTC, so were pooled and compared to pooled control rabbit plasma in the remaining tests. Hageman factor appeared to be normal and proaccelerin and proconvertin far higher in both normal and injected rabbit plasma than in human plasma. Only assays for AHF and PTC were significantly low in the injected group. As these assays are carried out on human substrates, *i.e.* plasmas from patients with hemophilia A or B, it is

TABLE I. Blood Clotting Factors in the Rabbits.

	Normal	Injected
Clotting time (min.)	5	6
Prothrombin consumption, %	95	95
Platelet count (per mm³)	566,000	662,000
Recalcification time (sec.)	120	120
Prothrombin time (sec.)	7.6	7.3
Prothrombin*	85	105
Proconvertin*	190	225
Proaccelerin*	200	300
AHF*		
on plasma	100	20
on fraction	100	120
PTC*		
on plasma	150	20
on fraction	100	100
Hageman Factor*	100	<100
Fibrinogen*	125	117

* As % of normal human level.

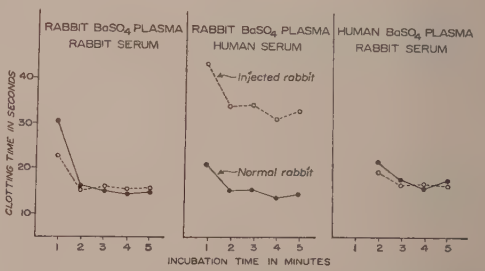

FIG. 1. Thromboplastin generation tests. All tests contained 0.2 ml rabbit platelets plus 0.2 ml 0.025 M $CaCl_2$. Substrate in all was normal human citrated plasma. Part I. 0.2 ml rabbit $BaSO_4$ adsorbed plasma (1-5) plus 0.2 ml rabbit serum (1-10) from normal and inj. rabbits. Part II. 0.2 ml normal or inj. rabbit $BaSO_4$ plasma (1-5) plus 0.2 ml human serum (1-10). Part III. 0.2 ml human $BaSO_4$ plasma (1-5) plus 0.2 ml normal or inj. rabbit serum.

TABLE II. Inhibition of Human Clotting Factors by Rabbit Plasma.

Test	Normal rabbit $BaSO_4$ plasma	Inj. rabbit plasma			
		$BaSO_4$	N*	PTC*	ProC*
Plasma Anticoagulant	0	2+	—	—	—
Anti-Prothrombin	0	tr	0	0	0
" Proconvertin	0	3+	0	0	3+
" Proaccelerin	0	0	0	0	0
" AHF	0	tr	0	0	tr
" PTC	0	4+	0	4+	tr
" Hageman Factor	0	0	0	0	0
" Fibrinogen	0	0	—		
" Thromboplastin	0	0	—		
Brain†	0	0	—		
Plasma	slight	slight	—		

* Samples of $BaSO_4$ treated rabbit plasma incubated 18 hr with equal volumes of normal human plasma (N), PTC deficient plasma (PTC) or proconvertin deficient plasma (ProC) and centrifuged at 20,000 rpm before testing.

† Tested on whole plasma.

possible that the assays are falsely low due to inhibition of PTC and proconvertin in the human substrates. That AHF and PTC in injected rabbits are actually normal is shown by the normal thromboplastin generation (Fig. 1, part 1) and by the assays on rabbit plasma fractions, prepared to contain the factor in question and to be free of inhibitors.

Inhibitory activity of rabbit plasmas to human clotting factors. Table II shows that the normal rabbit $BaSO_4$ plasma had no inhibitory effects other than a slight inhibition on formed plasma thromboplastin. $BaSO_4$ treated plasma from the injected rabbit prolonged normal human plasma recalcification time (plasma anticoagulant) and markedly inhibited human proconvertin and PTC. It also produced a slight diminution of prothrombin and AHF activity, but had no effect on proaccelerin, Hageman factor, fibrinogen or brain thromboplastin. In an attempt to separate the anti-PTC and anti-proconvertin activities of the injected rabbit plasma, equal volumes were incubated 18 hrs. (4°C) with normal human, PTC deficient, and hypoproconvertinemic plasmas. Tests on these mixtures showed that incubation with normal plasma removed all inhibitory effects, incubation with PTC-deficient plasma removed anti-proconvertin and incubation with proconvertin deficient plasma removed most of the anti-PTC.

Fig. 1 also demonstrates the inhibitory effects of the injected rabbit plasma. The first part of the figure shows that $BaSO_4$ plasma and serum from the injected rabbit generate plasma thromboplastin as effectively as those from the normal rabbit. In the second part the normal $BaSO_4$ rabbit plasma is effective when mixed with human serum, showing that rabbit AHF (and proaccelerin?) react normally with human PTC (and proconvertin?). On the other hand, the injected rabbit $BaSO_4$ plasma-human serum mixture does *not* generate thromboplastin. This can be interpreted either as lack of AHF in the injected rabbit or as inhibition of PTC (+ proconvertin) in the human serum. The third part of Fig. 1 shows that the activity of human $BaSO_4$ plasma (AHF + proaccelerin) is not inhibited by the injected rabbit serum.

Precipitin reactions. Normal rabbit plasma did not give precipitin reactions with human plasma. Plasma and serum obtained from the rabbits 12 days after the last injection of "PTC," either whole or $BaSO_4$ treated, gave markedly positive precipitin reactions with the "PTC" fraction, normal plasma or serum and PTC deficient plasma. Plasma obtained 12 days later still had marked anti-PTC and anti-proconvertin activity, but the precipitin reaction was far weaker and only a marked cloudiness without flocculation was observed. Table III shows that when this rabbit plasma was treated with $BaSO_4$ and then adsorbed with human plasmas of various types, the pre-

TABLE III. Precipitin Reactions.

Human material	Inj. rabbit plasma				
	7-11-56	7-23-56			
	$BaSO_4$	$BaSO_4$	N*	PTC*	ProC*
Normal plasma	4+	2+	0	+	+
" serum	4+	+	—	—	—
"PTC" fraction	4+	2+	—	—	—
PTC deficient plasma	4+	2+	0	0	+
Proconvertin deficient plasma	—	+	0	+	0

* See footnote Table II.

cipitin reactions were affected in the same way as the inhibitor reactions.

Discussion. The experiments illustrate clearly that the rabbit and human clotting factors studied have similar and interchangeable biological activities, but that the PTC and proconvertin of man and of rabbits are immunologically dissimilar. Following injections of a human serum fraction containing PTC and small amounts of proconvertin, rabbits developed inhibitors to these human factors but not to their own. Equally heavy precipitin reactions were observed when this rabbit antiserum was incubated with normal or with PTC deficient plasma, indicating the presence in the rabbit antiserum of antibodies other than anti-human PTC. Antiproconvertin was also identified. Separation of the antiproconvertin and anti-PTC activities was achieved by differential adsorption.

These experiments suggest that it may be possible to prepare antisera specific to the various human clotting factors. With sufficient purification of the antigens, antibodies of high specificity and in high titer may result which could be employed in assay methods independent of the formation and titration of thrombin.

Summary. Rabbits injected with human serum "PTC" developed inhibitors to human PTC and also to human proconvertin. These two activities could be separated by differential adsorption.

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Received July 31, 1956. P.S.E.B.M., 1956, v93.

Effect of Halogenated Aromatic Hydrocarbons on Proteins of Rat Tissues. (22779)

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The object of the present work is to study the effect of administration of various halogenated hydrocarbons on the protein content of rat tissues. Previous work(1-9) has indicated that the tissues are the most probable source of cysteine for the formation of mercapturic acids which are formed in the rat from various aromatic hydrocarbons. In view of the continuous exchange and renewal of tissue proteins at the expense of dietary amino acids, it appeared possible that the ingestion of aromatic hydrocarbons by the rat will interfere with the protein formation in the tissues by the interaction of the sulfhydryl groups with the administered hydrocarbons. The blocking of the sulfhydryl groups by the hydrocarbons should be reflected not only in the decrease in the protein content of the tissues but also in the decrease of the sulfhydryl and disulfide content of the proteins.

Methods. Albino rats of Sprague-Dawley strain were born and raised in the laboratory. Young rats, 30 to 38 days of age, weighed 63 to 133 g, older rats were of 86 to 186 days of age, weighing 176 to 366 g. In each case, age, sex, and weight of experimental and control rats were the same. Animals were housed in individual metabolism cages and water allowed *ad libitum*. Weights of animals and food consumption were recorded twice a week. Diet "D" had the following per cent composition: vitamin-free casein 8; sucrose 15; salt mixture 4 (U.S.P. XIV); Crisco 20; cod liver oil 5; corn starch 48. 200 mg of choline chloride and a complete mixture of all known vitamins in adequate amounts were mixed with the diet. Diet "A" contained 27% of casein and 29% of starch, the rest of the ingredients were in the same concentration as in Diet "D." The nitrogen content of both diets was determined. *Halogenated aromatic hydrocarbons.* Phenacyl bromide, p-chlorobromobenzene, benzyl chloride, and naphthalene tetrachloride were commercial products, and the purity of the products was established in the laboratory. As previously recorded by others(7), large amounts of the compounds sharply reduced food intakes, caused a rapid loss in weight, leading eventually to death of the animals. On autopsy, pulmonary hyperemia, hemorrhagic kidneys and occasionally hemorrhages in the pericardium were observed. No other abnormal histological changes in the lungs or kidneys were observed. The quantities of hydrocarbons fed to rats were reduced to amounts which could be tolerated by the animals. With the intraperitoneal injections of naphthalene tetrachloride, the dose was 100 mg (as a water suspension containing 1% "Tween 80")/100 g weight. *Analytical methods.* The nitrogen content of diets, excreta, and various organs was determined by the Conway ultramicro Kjeldahl diffusion method. In nitrogen balance studies the urine collected daily, and feces, collected separately, were analyzed twice a week. All determinations were made in at least triplicate. The results of nitrogen balance studies were charted according to Reifenstein *et al.*(10). The protein content of tissues was calculated as suggested by Addis *et al.*(11,12). In each tissue water content and nitrogen, the latter in a homogenate, were determined. Whole blood was used for the nitrogen analysis. Animals were anesthesized under ether, blood samples were taken from abdominal aorta, and organs removed and thoroughly washed of all blood. Organs of rats from the same experimental groups were pooled, and analyses made on pooled samples. The sulfhydryl and disulfide groups in blood were determined in the formic acid-hydrochloric acid digests employing the procedure of Kolb and Toennies(13). Total circulating blood volume was determined with T-1824 (Evans-Blue). The ratio of muscle to body weight had been previously determined on 12 adult male rats, with

TABLE I. Effect of Halogenated Aromatic Hydrocarbons in Growing Rat.*

No. and sex of rats	Hydrocarbon, % in diet	Intake diet	Days	Avg intake per day, g	Avg daily change, g
6 ♀	Phenacyl bromide, 0.5	D	38	5	−.85
6 ♀	p-Chlorobromobenzene, 1.0	D	52	8	.28
6 ♀	None	D	45	10	1.16
12 ♂	N. T., 0.25	D	30	6	−.70
12 ♂	None	D	30	6	.47
12 ♂	N. T., 1.0	A	30	21	3.46
12 ♂	None	A	30	21	5.60
6 ♀	N. T. in glycerin, 0.5	A	13	12	1.53
6 ♀	None	A	13	12	2.81
6 ♀	N. T. in glycerin, 0.25	D	19	7	−.84
6 ♀	None	D	19	7	1.15

* "N. T." denotes naphthalene tetrachloride. Intramusc. injections of N. T. emulsions in water or olive oil into rats fed diets D or A did not significantly affect growth rates, due, perhaps, to poor absorption of the hydrocarbon from site of inj.

a mean of 41%, the coefficient of variation $\left(\frac{\text{Standard Deviation}}{\text{Mean}}\right)$ being 10.5%. Weight of skeleton was calculated from body weight using the data of Donaldson(14).

Results. The data in Table I show that phenacyl bromide, p-chlorobromobenzene, or naphthalene tetrachloride inhibited growth of rats which ingested the 8% casein diet. Similar results, not reported here, were obtained on mice which were fed benzyl chloride, p-chlorobromobenzene, bromobenzene, or naphthalene. No significant inhibition of growth was obtained in young rats which ingested the same hydrocarbons with the 27% casein diet, although some inhibition of growth was observed in rats which were administered naphthalene tetrachloride as glycerin emulsion. The results in Table II show that the

TABLE II. Response of Adult Rats to Naphthalene Tetrachloride.

No. of rats	Naphthalene tetrachloride	Diet	Intake days	Avg daily intake	Avg daily wt change, g
6	0.25% in diet	D	58	10	−1.15
6	*Idem*	D*	10	14	4.55
5	"	D	58	12	.58
5	"	D*	10	13	2.40
6	1% in water, intraper.	A	15	10	−7.51
6	None	A	16	20	2.71
6	In glycerin, intraper.	A	17		−1.05
6	None	A	43		.62

* 0.12% of L-cystine in diet.

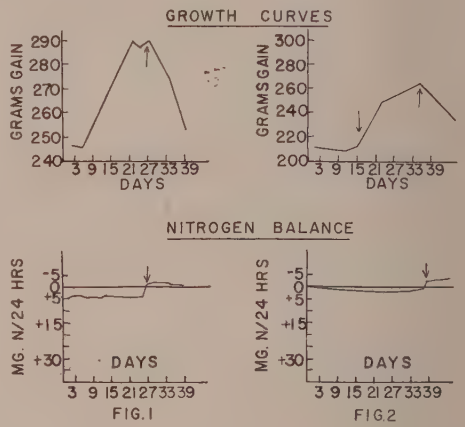

FIG. 1 and 2. Effect of naphthalene tetrachloride on gain in wt and nitrogen balance in rats. Data represent avg results obtained on 6 male rats 128-132 days old. Upper curves are wt curves; lower curves represent nitrogen balance. Animals fed same diet for 14 days before collection of excreta. Animals represented by left 2 curves were fed Diet A plus 0.25% naphthalene tetrachloride. Arrow indicates time at which animals were continued to be fed Diet A but naphthalene tetrachloride was inj. intraper. The right 2 curves represent animals which were fed Diet D plus 0.25% naphthalene tetrachloride. First arrow indicates time at which L-cystine was added to diet. Second arrow indicates time at which naphthalene tetrachloride was inj. intraper. to rats while feeding of Diet D was continued.

intraperitoneal injection of naphthalene tetrachloride into adult rats produced significant loss in weight, even when the animals ingested the 27% casein diet. The results on nitrogen balance in these animals are summarized in Fig. 1 and 2. The animals re-

TABLE III. Protein of Tissues of Rats Which Received Naphthalene Tetrachloride Intraperitoneally for 12 Days, Calculated in g per Rat per 310 cm² of Body Surface.*

	Group 1 Diet A	Group 3 Diet A + N.T.	Change in protein content, %	Group 2 Diet D	Group 4 Diet D + N.T.	Change in protein content, %
Liver	1.57	1.49	− 5	1.41	1.56	11
Kidneys	.26	.22	−14	.22	.24	9
Digestive tract	.88	.50	−42	.72	.67	− 7
Muscle	13.85	8.40	−39	12.87	10.89	−13
Lungs	.13	.11	−15	.10	.13	30
Spleen	.07	.04	−41	.05	.04	−20
Heart	.11	.08	−27	.12	.09	−25
Blood	1.99	1.22	−38	1.69	1.26	−25
Pelt	8.02	7.09	−11	6.21	4.25	−31
Skeleton	1.55	1.34	−13	1.46	1.42	− 3
Carcass	7.88	5.57	−29	7.30	5.56	−23
Total	36.31	26.06	−23	32.13	26.10	−19

* The data were obtained on pooled tissues of animals in each group. Nitrogen balance of these animals during the 12 days of N. T. inj. is indicated in Fig. 1 and 2 by the respective arrows at extreme right portion of figures.

mained in positive nitrogen balance while ingesting diet "A" or "D" with naphthalene tetrachloride. However, when naphthalene tetrachloride was injected a definite negative nitrogen balance was observed. After 12 days of injection of naphthalene tetrachloride the animals were sacrificed, the organs were analyzed for protein content, and the results are shown in Table III. It will be noted that a loss of protein has occurred in all tissues examined, except in animals which ingested diet "D." In the latter case an increase in the protein content of the liver, kidneys, and lungs was noted. The data in Table IV suggest that a decrease in the sulfhydryl and particularly in the disulfide content of the rat blood has occurred during the injection of naphthalene tetrachloride.

Discussion. Loss in weight of rats ingesting hydrocarbons together with the 8% casein diet does not appear to be due entirely to the drop in food consumption, as was indicated by paired feeding experiments. The prompt resumption of growth on incorporation of L-cystine into the hydrocarbon-containing diet suggests interference of the hydrocarbon with the utilization of cystine (cysteine). This inference is supported by the apparent decrease in the -SH and S-S content of the blood during the intraperitoneal administration of naphthalene tetrachloride. It is of interest to note that Tsuji(15) and Nakashima(16) observed a decrease in the free and "bound" cysteine of the eye lens proteins as well as of glutathione of the liver and eye lens of rabbits which were fed naphthalene. As has been established, naphthalene undergoes *in vivo* conjugation with cysteine and is in part excreted in the urine of animals as the naphthalene mercapturic acid. It is possible that naphthalene tetrachloride is similarly metabolized in part to a mercapturic acid derivative. The data further suggest that in the course of metabolic disposal of the hydrocarbons examined an interference with a normal metabolism of proteins in various tissues takes place which is minimized either by a high protein content of the diet or by supplementary cystine. Binding of various hydrocarbons to the tissue proteins has been emphasized in recent years. We have no direct evidence that the hydrocarbons which we examined in this study combine directly with the tissue pro-

TABLE IV. Sulfhydryl and Disulfide Content of Blood of Rats Which Receive Intraperitoneally Naphthalene Tetrachloride for 12 Days.*

Diet and hydrocarbon	Sulfhydryl for 310 cm² of original body surface	Disulfide for 310 cm² of original body surface
A	1.36	19.52
A + N. T.	.68	5.22
D	1.12	12.93
D + N. T.	.71	6.75

* Sulfhydryl and disulfide expressed in mg as cysteine and cystine respectively/rat.

teins, although the possibility exists that the hydrocarbons interacted with the -SH or S-S groups of the proteins in a manner which prevented the detection of these groups by the analytical procedures which we employed. The binding of the hydrocarbons to the proteins, particularly in liver, could conceivably be reflected in the alteration of their metabolic availability to other organs and in the protein content of these organs.

Summary. Phenacyl bromide, p-chlorobromobenzene, or naphthalene tetrachloride inhibit growth of rats which ingested an 8% casein diet. The inhibition was alleviated by supplementary cystine, or by a 27% casein diet. Nitrogen balance remained positive in rats which ingested the 27% casein diet or the 8% casein diet which was supplemented with cystine. Intraperitoneal injection of naphthalene tetrachloride into adult rats on the high or low protein diet induced a negative nitrogen balance, accompanied by a decrease in the -SH and S-S content of the whole blood and in the protein content of practically all tissues. The implication of these observations is discussed in terms of possible interference of the hydrocarbons with the protein metabolism of the rat via direct binding of the hydrocarbons to the proteins through the -SH or S-S groups.

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Received October 13, 1955. P.S.E.B.M., 1956, v93.

Coagulation-Promoting and Inhibitory Properties of Modified Thrombin Preparations.‡ (22780)

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The fibrinogen-clotting activity of thrombin is known to be destroyed by heating above 60°C(1). As will be shown here, such heated preparations have profound effects on various phases of the coagulation mechanism.

Materials and methods. Tropical thrombin;* bovine fibrinogen, Fraction I;† human plasma; bovine plasma; human platelet material; degradation products of thrombin. Human plasma was prepared from normal blood which had been treated with a solution of sodium oxalate (1 part of 0.1 M oxalate solution in water to 9 parts of blood) and was centrifuged at 32,000 g for 30 minutes at room temperature. Bovine plasma was prepared in a similar manner. Human platelet material was prepared by the method described previously(2). Inhibitors of thrombin were prepared by heating solutions of thrombin in isotonic saline in the waterbath for varying periods at temperatures ranging from 40°C to 100°C. In most of the work reported here, the material studied was ob-

‡ This investigation was supported by the Atomic Energy Research Contract AT(30-1) 1275 and by Grant No. C937 from the U. S. Public Health Service.

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† Armour and Co., Chicago, Ill.

FIG. 1. Ultracentrifugal pattern of supernatant obtained from boiled thrombin preparation.

tained by heating thrombin at 100°C for 30 minutes. The thick coagulum which formed was removed by centrifugation. The supernatant (B.T.) was precipitated with 9 volumes of 95% ethyl alcohol. The white precipitate (A.T.) was collected by centrifugation, dried *in vacuo* and redissolved in water, physiological saline or imidazole buffer at a pH of 7.4. The activity obtained by treating one unit of thrombin as described above will be referred to as one unit of B.T. or A.T., respectively.

The following determinations were carried out on plasma to which B.T., A.T. or a blank (saline or buffer) had been added: recalcification time according to a modification(3) of the method of Quick(4); thromboplastin generation test(5) modified as described previously(6). The thrombin time was determined by forcefully ejecting 0.1 cc of thrombin solution into test tube (7 mm) containing 0.1 cc of buffer or solution of heated thrombin preparation and 0.2 cc of a solution of 400 mg % fibrinogen in imadazole buffer. The first appearance of a fibrin thread was taken as the end-point. Thrombin generation, estimated by the method previously described (7), was modified in the following manner. 4 cc of a 0.25 M solution of calcium chloride in water were added to 2 cc of oxalated plasma at 37°C. Aliquots of 0.1 cc of this mixture were aspirated into tuberculin syringes containing 0.1 cc of B.T., A.T. or a

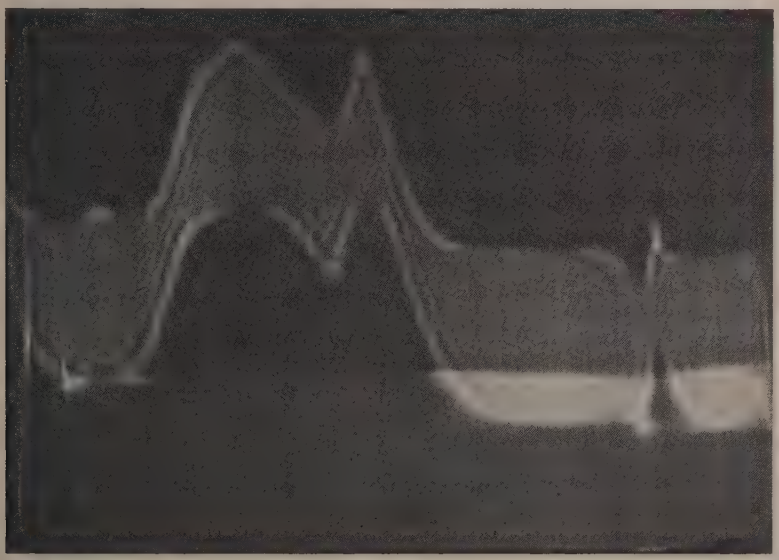

FIG. 2. Free boundary electrophoretic pattern of supernatant obtained from boiled thrombin preparation.

TABLE I. Effect of Varying Concentration of A.T. on Recalcification Time of Bovine Plasma.

A.T. units*	Clotting time, sec.
0	157
1	165
10	172
100	176
500	192
1000	265
5000	600
10,000	600

* A.T. = Alcohol precipitate. One unit of A.T. is the amount obtained from one unit of thrombin.

blank at minute intervals. The contents of the syringe were ejected forcefully into test tube (7 mm) containing 0.2 ml of a solution of 400 mg% fibrinogen in buffer. The tube was agitated by manual rotation at 37°C. The first appearance of fibrin strands was recorded as the end-point.

Results. Chemical characteristics: Activities in supernatant (B.T.) obtained after centrifugation of heated bovine thrombin solution, and the precipitate (A.T.) obtained by adding ethyl alcohol to B.T. were non-dialyzable, contained peptide bonds (Biuret test), carbohydrate (Orcinol test) and amino sugars (Morgan Elson reaction). Free reducing groups were not detected. In the analytical ultracentrifuge, a uniform peak is observed (Fig. 1) which differs markedly from that given by the starting material. On free boundary electrophoresis a pattern is obtained which resembles the starting material in terms of the general relation of the major peaks to each other, but the rate of migration is considerably reduced in the modified preparation (Fig. 2).

The yield of A.T. amounts to approximately 5% of the weight of the starting material.

Coagulation studies: High concentrations of heated thrombin or the alcohol precipitate inhibited the clotting of recalcified bovine plasma. Acceleration was not observed at any concentration of these materials (Table I).

Coagulation of recalcified human plasma, however, was accelerated by B.T.‡ or A.T.§ except at high concentrations, which may cause inhibition. Low concentrations were ineffective. (Table II). It appears that these effects are more marked with stored plasma.

TABLE II. Effect of Varying Concentrations of A.T. on Recalcification Time of Human Plasma.

Duration of storage of plasma	Control (Imidazole buffer)	500 units* of A.T.†	250 units of A.T.	125 units of A.T.	62.5 units of A.T.	31 units of A.T.
	(sec.)					
Fresh plasma	220	485	310	175	210	240
Stored plasma (24 hr)	185	215	100	97	125	155
" " (1 wk)	240	305	270	186	197	236
" " (2 ")	190	115	115	90	130	150
" " (3 ")	160	118	115	110	128	—

* 1 unit of A.T. is the amount obtained from 1 unit of thrombin.
† Alcohol precipitate.

It can be seen from Table III that the accelerating activity of B.T.‡ decreases as the lengths of time for which it is kept at 100°C increases. Addition of calcium ion leads to partial reactivation.

TABLE III. Effect of Varying the Period for Which Thrombin Is Heated at 100°C on Activity of A.T. in the Clotting of Human Plasma. (Control without addition of thrombin derivative—347 seconds.)

Time of heating thrombin at 100°C, min.	Clotting times (sec.) Without added calcium	With added calcium
0	1	1
5	85	30
10	116	57
20	600	105
30	600	132
60	600	210

‡ Supernatant obtained from heated thrombin preparation.

§ Alcohol precipitate from supernatant of heated thrombin preparation.

TABLE IV. Effects of Pre-incubating Human Plasma with 300 Units of A.T. for Varying Periods Prior to Addition of Ca^{++}.

Time of pre-incubation, min.	Clotting times, sec.
0	190
2	18
4	9
6	5
10	2

Rate of thromboplastin generation was increased by B.T.‡ or A.T.§ except at high concentration of these materials, which caused inhibition. (Table V).

TABLE V. Effect of Varying Concentration of A.T.* on Generation of Thromboplastin in Human Plasma.

Time of incubation of mixture, min.	Control	A.T.* 300 units†	A.T. 60 units	A.T. 10 units
	(sec.)			
1	88	58	42	50
3	43	30	12	12
4	—	—	11	11
5	13	18	11	11
6	10	17	11	11
7	10	16	11	12
8	11	15	12	—
9	—	16	—	—

* Alcohol precipitate obtained from supernatant of heated thrombin preparation.
† One unit of A.T. is the amount obtained from one unit of thrombin.

Inhibitory effects of heated thrombin preparations on coagulating activity of native thrombin. When bovine thrombin was used in "thrombin time" determinations on human and bovine plasma, its coagulating activity was inhibited by B.T.‡ and A.T.§

TABLE VI. Effect of Varying Concentration of A.T.* on Activity of Bovine Thrombin Added to Human and Bovine Plasma.

A.T. units*	Human plasma, sec.	Bovine plasma, sec.
10,000	—	255
5000	—	160
1000	47	28
500	38	25
100	29	19
10	25	16
1	25	16
0	25	16

* Alcohol precipitate obtained from supernatant of heated thrombin preparation. One unit of A.T. is the amount obtained from one unit of thrombin.

In the thrombin generation test, B.T.‡ and A.T.§ produced inhibition in the bovine test system, but not in the human system. Representative findings are tabulated in Table VII.

TABLE VII. Effect of A.T. on Activity of Thrombin Generated in Bovine and Human Plasma.

Time of incubation, min.	Bovine plasma		Human plasma	
	Control	500 units* A.T.† added	Control	500 units* A.T.† added
	(sec.)			
2	205	—	212	240
3	63	—	135	173
4	30	313	43	38
5	30	240	32	30
6	24	176	30	26
7	24	99	30	25
8	24	72	25	23
9	25	65	25	22
10	29	69	30	25
12	—	75	30	27

* One unit of A.T. is the amount obtained from one unit of thrombin.
† Alcohol precipitate obtained from supernatant of heated thrombin preparation.

Discussion. The exposure of thrombin to temperatures above 60°C has been found to lead to loss of its ability to clot fibrinogen. The data presented here refer to the alcohol precipitate obtained from the supernatant of heated bovine thrombin preparations, but essentially the same results were obtained when the supernatant prior to the addition of alcohol was used. The data indicate that the redissolved alcohol precipitate (AT) obtained from heated thrombin solution has a number of effects on the various stages of the coagulation mechanism. None of these effects is due to a true thrombin activity, as evidenced by failure to clot fibrinogen. 1. The effects of the alcohol precipitate obtained from heated thrombin preparations on the recalcification time may be related to the increased rate of the generation of thromboplastin. This aspect of thrombin activity appears to differ qualitatively from its coagulant effects on fibrinogen and is more stable than the latter. 2. The effects of incubation of heated thrombin preparations with plasma prior to the addition of calcium are not understood. 3. The alcohol precipitate obtained from solutions of heated thrombin preparations inhibited bo-

vine thrombin. This inhibitor appears to be different from the antithrombic activities described so far(8). It should be noted that the considerably lower concentrations of thrombin derivatives prepared from bovine thrombin were sufficient to inhibit the latter, than were required to inhibit thrombin. This species-specific inhibitory effect may be related to differences between bovine and human thrombin and to the ease with which the respective enzymes can be inhibited by the same inhibitor. On the other hand, the possibility may also be considered that the modification of an enzyme may result in a derivative which will inhibit preferentially the specific enzyme from which it has been prepared.

The heated thrombin preparations and the redissolved alcohol precipitates have been administered intravenously to rabbits and guinea pigs without toxic or thromboembolic sequelae.

Summary. An alcohol precipitate obtained from heated bovine thrombin preparations promoted the early phases of the coagulation mechanism, but did not clot fibrinogen in the manner of the unheated preparation. These materials inhibited bovine thrombin at lower concentrations that human thrombin in the clotting of fibrinogen.

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Antifibrinolytic Activity of Derivatives of Fibrinolysin.‡ (22781)

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Fibrinolysin and its inhibition and activation have been intensively investigated in recent years in relation to coagulation and hemostasis. The clinical investigation of bleeding patients(1) as well as the fractionation of lyophilized human platelet material suggested the presence of an inhibitor of fibrinolysin in these materials. Antifibrinolysin, moreover, has been demonstrated previously in bovine platelets(2).

In the search for specific inhibitors of fibrinolysin, an attempt was made to produce an inactive derivative which would specifically inhibit the enzyme.

Materials and methods. Bovine fibrinolysin* was suspended in water, saline or buffer (imidazole pH 7.2), and heated at 90°C for periods varying from 5 to 30 min. Coagula formed during the heating were removed by centrifugation at 3,000 g for 10 min. The supernatant (M.F.) was precipitated with 3 volumes ethyl-alcohol to yield a white, water-soluble material (MFP). The amount of MFP obtained was approximately 4.5% of the weight of the starting material. The fibrin-plate method previously described (3) was used for the determination of fibrinolytic activity. The method was modified, however, in regard to times and temperatures of incubation. Human fibrinolysin activity was obtained by activating normal human serum with Streptokinase (Varidase).*

Results. The soluble material remaining after heating bovine fibrinolysin at 90°C was

‡ This investigation was supported by the Atomic Energy Research Contract AT(30-1) 1275 and by Grant No. C 937 from the U. S. Public Health Service.

* We acknowledge with gratitude the generous sample received from Dr. Eugene C. Loomis, Parke Davis and Co., Detroit, Mich.

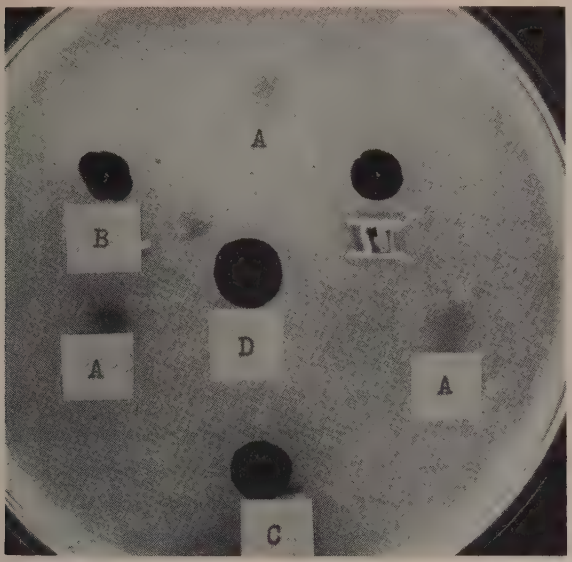

FIG. 1. A. 10 mg/cc active fibrinolysin solution mixed 1:1 with MF derived from 10 mg/cc of fibrinolysin. B. 10 mg/cc active fibrinolysin diluted 1:1 with saline. C. 10 mg/cc active fibrinolysin (undiluted). D. 20 mg/cc active fibrinolysin (undiluted).

insoluble in alcohol and gave a strongly positive orcinol reaction, indicating the presence of carbohydrate.

Both the supernatant (M.F.) as well as the material precipitated from it by alcohol (MFP), inhibited the activity of fresh fibrinolysin, as illustrated in Fig. 1.

In another experiment with MFP† the fibrin plates were incubated for 48 hours. Lysis of fibrin started at the point of application of fibrinolysin and spread to other areas of the plate. The area on which MFP had been applied (Fig. 2 and Fig. 3) was protected from lysis, while despite some lysis at the center of the site of application of a mixture of active and modified fibrinolysin, the surrounding fibrin was not lysed. Qualitatively similar results were also obtained with the supernatant (MF) obtained from heated fibrinolysin.

The effect of heating for various periods of time was investigated. Optimal inhibition was obtained with materials heated for 5 to 10 minutes, while heating for more than 20 minutes tended to decrease the inhibitory activity.

† Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

When modified bovine fibrinolysin was tested against human fibrinolysin (*i.e.*, serum activated with Streptokinase), fibrinolytic activity was not antagonized (Table I).

Discussion. Products obtained by heating bovine fibrinolysin preparations were found to inhibit the activity of the unmodified enzyme. The same material did not appear to affect the fibrinolytic activity of human serum treated with streptokinase. It should be noted that the successful formation of inhibitors by heat denaturation varied with the nature of the starting material and the procedures employed, in a manner not understood at the present time.

Analogous inhibitory phenomena have been

TABLE I. Effect of MFP on Human Serum Fibrinolysin.

	Avg diameter of lysed area (mm)
Activated human serum 1:1 with 20 mg MFP*/cc	16.5
Idem, with 10 mg MFP/cc	16.0
" with saline	16.2

* MFP refers to the alcohol precipitate, *i.e.*, 20 mg of dried precipitate/cc of physiological saline.

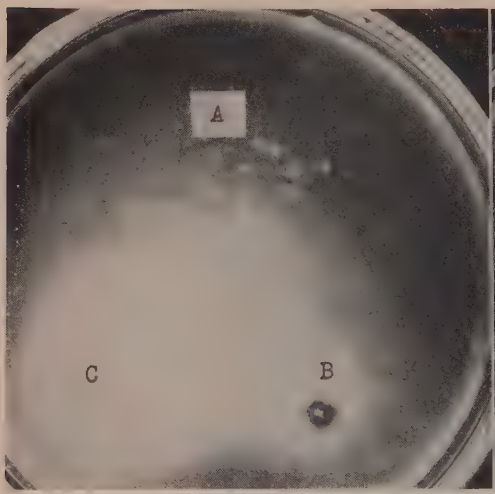

Fig. 2

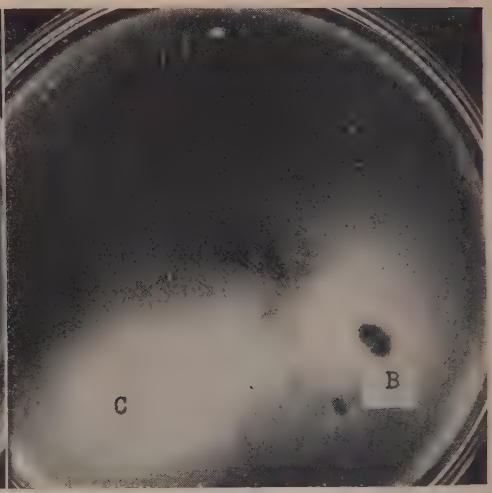

Fig. 3

FIG. 2. A. Fibrinolysin, 20 mg/cc, diluted 1:1 with saline. B. Fibrinolysin, 20 mg/cc, diluted 1:1 with MFP,* 20 mg/cc. C. MFP, 20 mg/cc.

FIG. 3. A. Fibrinolysin, 20 mg/cc, diluted 1:1 with saline. B. Fibrinolysin, 20 mg/cc, diluted 1.1 with MF† obtained from 20 mg fibrinolysin/cc. C. MF, 20 mg/cc.

* 20 mg of dried alcohol precipitate were dissolved in 1 cc of physiological saline.

† MF refers to supernatant obtained from heating a solution of 20 mg of fibrinolysin/cc.

observed in parallel studies of thrombin and in preliminary studies with a number of other enzymes which are not related to the coagulation mechanism.

The failure of an inhibitor derived from a bovine fibrinolysin preparation to affect human fibrinolysin activity (Table I) is of interest. Studies are in progress to determine the species-specificity of this inhibitory effect, particularly in view of the known differences between functionally similar enzymes derived from different tissues and different species.

Summary. Derivatives of bovine fibrinolysin have been prepared which inhibited the enzyme from which they are derived, but did not inhibit human fibrinolytic activity.

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Received August 2, 1956. P.S.E.B.M., 1956, v93.

Effect on Experimental Avian Atherosclerosis of Dietary Oils and Gallogen. (22782)

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(Introduced by Camillo Artom)

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The diethanolamine salt of the mono-(+)-camphoric acid ester of α, 4-dimethylbenzyl alcohol (Gallogen) has been reported(1) to impede serum and tissue cholesterol elevations in the cholesterol-fed cockerel. Recently, clinical(2-7) and experimental(8-12) aspects of the relationship between the degree of unsaturation of dietary fats and their effects on serum cholesterol have begun to be explored. The experiment reported here was designed to show what effect the feeding of fats of various degrees of unsaturation, singly and with Gallogen, would have on atherogenesis in the cholesterol-fed cockerel.

Method. The experimental arrangements and procedure were as previously described (1). Twenty-one-day-old cockerels were placed on a diet of Purina Growing Mash and 1% Cholesterol and divided into 6 groups, which received additional materials as follows: Group I, 8% Peanut Oil;* Group II, 8% Coconut Oil;† Group III, 8% Sardine Oil;‡ Group IV, 8% Peanut Oil, 0.1% Gallogen; Group V, 8% Coconut Oil, 0.1% Gallogen; Group VI, Sardine Oil, 0.1% Gallogen. An edible antioxidant, butylated hydroxyanisole (Eastman), 0.01%, was added to the sardine oil and, as a control, to the coconut oil. The diets were mixed every 2 weeks, except for the sardine oil, which was refrigerated and mixed weekly.

Results. Food consumption was equal for all the groups. During the experimental period (Feb.-June) there were 2 deaths. One occurred in Group IV during the seventh week and was of unknown cause; the other was in Group I during the eleventh week and was due to anemia complicated by endocarditis.

TABLE I. Mean Serum Cholesterol Levels in mg per 100 ml.

Wk	Group* I	II	III	IV	V	VI
0	151	151	155	161	149	147
1	497	660	532	511	618	317
2	879	1025	769	725	889	524
3	939	850	910	798	1014	598
4	1113	1002	996	891	784	556
5	1039	934	974	877	814	534
7	1177	1057	886	1058	834	489
9	1081	1061	864	927	919	476
11	1252	1106	469	938	1143	245
12	1475	1154	633	1076	1259	366
13	1331	1001	838	1029	1264	485
14	1304	1118	1123	1014	1046	447
15	1617	1273	1219	1097	1092	568
16	1642	1339	1372	1143	1054	623

* Groups I and II had 8 birds, the remaining groups had 9 each.

The averages of weekly (or fortnightly) individual serum total cholesterol determinations are given in Table I. Unfortunately, 5 days before the blood samples were obtained at the end of week 11, the supply of sardine oil was exhausted. The two groups on sardine oil were fed regular mash until their experimental diet could be resumed at the end of week 11. It can be seen that the serum cholesterol levels of both groups receiving sardine oil declined sharply during this time, but rose again when the experimental ration was resumed. This interruption, although relatively brief, causes the succeeding serum cholesterol figures for Groups III and VI to be somewhat equivocal. The Gallogen-sardine oil group did not rise much above the level attained by week 9, but the sardine oil-alone group continued to rise sharply until the experiment was ended.

Although there were no striking differences among the average serum levels of Groups I, II, and III, the group receiving sardine oil was almost consistently the lowest of the 3, by a small margin.

* Obtained from Welch, Holme, and Clark Co., New York City, Iodine no. 95-100.

† Obtained from Welch, Holme, & Clark Co., New York City, Iodine no. 9-11 m.p. 24°C.

‡ Obtained from Pacific Vegetable Oil Corp., Gardner color 11, Iodine no. 190-200.

TABLE II. Post-Treatment Aortic and Hepatic Cholesterol Concentrations, and Body Weights.

Group	Aortic cholesterol (mg/g dry wt)*	Hepatic cholesterol (mg/g dry wt)*	Avg body wt (g)
I	19.7 ± 4.8†	81.9 ± 8.4	1833
II	20.0 ± 7.6	74.1 ± 17.8	1561
III	20.9 ± 5.8	76.5 ± 17.0	1703
IV	14.6 ± 3.6	78.2 ± 16.5	1727
V	23.9 ± 8.3	74.7 ± 25.9	1637
VI	10.7 ± 3.6	49.7 ± 12.1	1686
Normal (12 wk, from ref. 1)	7.6 ± 1.2	20.5 ± 3.0	—

* The dry wt/wet wt ratio for aorta and liver is 0.25 and 0.42, respectively.

† ± Stand. dev. $\sigma = \sqrt{\frac{\Sigma d^2}{N}}$.

The addition of Gallogen to the rations containing the various oils resulted in a decrease in the serum cholesterol in each case. This decrease was small and inconsistent in the case of the coconut oil (saturated), larger in the case of peanut oil (moderately unsaturated), and quite distinct for sardine oil (highly unsaturated), with the reservations mentioned above.

The body weights at necropsy and the results of the tissue analyses are detailed in Table II. It is apparent that the aortic cholesterol levels were essentially the same for the 3 fat-alone groups, but were again inversely proportional to the unsaturation of the fats when these were co-fed with Gallogen. Hepatic cholesterol was affected markedly only in the group fed both sardine oil and Gallogen. Body weight was highest in Groups I and IV, receiving peanut oil, and lowest in Groups II and V, receiving coconut oil.

It is noteworthy that a separately caged group, not described in this paper but receiving a control atherogenic diet plus a drug having no apparent effect, paralleled closely the serum values of Group I (and their weekly variations) throughout the experimental period, and had aortic and hepatic cholesterol values averaging 19.1 and 84.4 mg/g, respectively. This serves to illustrate the degree of precision attained in this experiment.

Summary. 1. Dietary fats of varying degrees of saturation were no different from each other with respect to their effect on aortic or hepatic cholesterol levels of cholesterol-fed cockerels and only slightly with respect to serum levels. 2. When 0.1% Gallogen was added to the diet, serum and aortic levels were in proportion to the degree of saturation of the fat fed.

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Received August 27, 1956. P.S.E.B.M., 1956, v93.

Tryptophan Content of Casein as Determined by a Rat Growth Method.* (22783)

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Extreme variations in values for the tryptophan content of casein have appeared in the literature(1). These have ranged from 1.68%, obtained by applying a colorimetric method to the intact protein(2) to 0.92% listed in(3). Using the colorimetric method referred to above, analysis of alkaline hydrolysates gave a value of 1.24%(4).† The latter value corresponds closely to those found by microbiological assay of alkaline hydrolysates, 1.20%(5), 1.24%(6), 1.18-1.24%(7), or enzyme hydrolysates 1.28% (6), 1.31%(8), and 1.20%(1). Lower values can be attributed to the extreme ease with which tryptophan is destroyed by hydrolysis procedures or to incomplete hydrolysis in the case of enzymatic methods. The higher values may result from lack of specificity of the chemical methods which have been used. In this connection, it is interesting to note that a bioassay with *Tetrahymena geleii*(9), in which preliminary hydrolysis is not required, has given results which agree closely with those of most microbiological assays.

In view of this extreme variation, probably due largely to difficulties in hydrolysis, it seemed desirable to apply an animal assay to this problem. Such assays have been widely used for most essential nutrients and it seemed particularly applicable to tryptophan, since the report by Oesterling and Rose(10) indicated that the rat's weight gain is related linearly to the L-tryptophan dosage, when the latter is varied from 0.10 to 0.15% of the diet. The chief source of error in this assay method might result from incomplete hydrolysis of the protein with accompanying incomplete absorption of tryptophan from the small intestine. This would tend to give low values but the same errors would be encountered whenever casein is employed in rat feeding experiments. On the other hand, high values might result if the free tryptophan used as the standard were not utilized as well as that released from the digested casein.

Methods. Female weanling rats weighing 55 to 65 g were obtained from the local stock colony. The experimental period was 4 weeks during which time the animals were housed in individual cages in an air-conditioned room and given food and water *ad libitum*. Weekly weight gains and daily food consumption were recorded. The basal diet contained 15% acid-hydrolyzed casein. This preparation, previously described(11), had the following amino acid composition: phenylalanine, 4.0%; histidine, 1.1%; methionine, 2.7%; arginine, 2.6%; valine, 6.2%; leucine, 8.0%; threonine, 2.9%; isoleucine, 4.7%; lysine, 5.9%; total nitrogen, 13.28%. The remaining components of the basal diet were: 4% Salts IV(12), 2% vitamin mixture, 0.1% L-histidine, 0.2% L-cystine, 5% corn oil and sucrose to make 100%. The vitamin mixture provided the following quantities per kg of diet: thiamine • HCl 2 mg, riboflavin 3 mg, pyridoxine • HCl 2.5 mg, calcium pantothenate 20 mg, choline chloride 1 g, inositol 100 mg, biotin 0.1 mg, folic acid 0.2 mg, vit. B_{12} 17 μg, and niacin 25 mg. Levels of L-tryptophan from 0.04 to 0.15% or of casein from 4 to 9% were incorporated into the basal diet with suitable reductions in the acid-hydrolyzed casein to maintain the nitrogen level of the diet at 1.99%. The L-tryptophan used was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. The casein employed in these experiments was vitamin-free Labcocasein (Borden Co., N. Y.) and con-

* This study was supported by grant from U.S.P.H.S., R.G., A-801.

† The recent use of the higher values in the design and interpretation of nutritional experiments prompted the studies reported here. See Ebisuzaki, K., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1952, v198, 63, and Hopper, J. H., and Johnson, B. C., *J. Nutrition*, 1953, v56, 303.

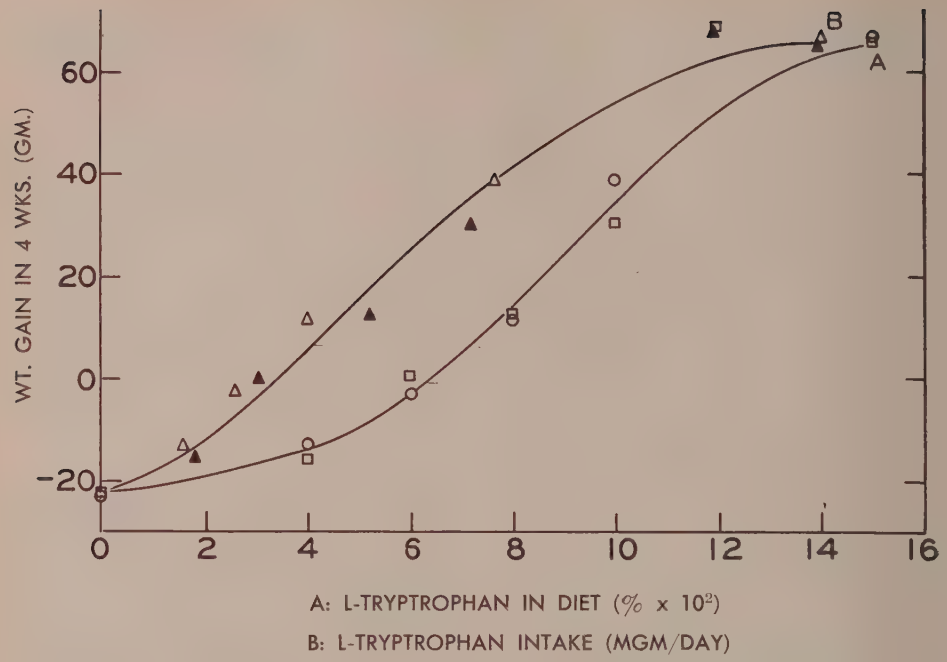

FIG. 1. Standard dose-response curves for tryptophan expressed as % of diet and as intake in mg/day.

tained 14.64% nitrogen on a moisture-free basis.

Results. Fig. 1 presents standard curves representing the data from two experiments. From the daily food consumption and the level of tryptophan in each diet, it was possible to calculate the daily L-tryptophan intake which is plotted against the growth rate in curve B. Curve A was constructed by plotting the growth rate versus the level of L-tryptophan in the diet. The experimental points from both studies are presented, but since the results were in good agreement, single standard curves were drawn. In each experiment the increase in body weight during 4 weeks for each level of casein was measured; these growth rates in conjunction with curve A provide the basis for the calculation of the per cent tryptophan in these diets and, from this, the per cent tryptophan in the casein (Column A, Table I). The most nearly linear portion of the dosage-response curve (A), which gives the most reliable value for the tryptophan present in casein, lies between 0.05 and 0.12% L-tryptophan. Food consumption data on the same animals were

TABLE I. L-Tryptophan Content of Casein.*

Exp.	Casein in diet, %	Avg wt gain 4 wk, g	Tryptophan in casein (%) A†	B†
I	4	− .5	1.56‡	1.82‡
	6	7.5	1.22	1.32
	8	36.5	1.28	1.33
		Avg	1.35 ± .08§	1.49 ± .29
II	5	−4.0	1.16	1.33
	6	8.5	1.23	1.28
	7	14.5	1.06	1.09
	8	38.0	1.29	1.20
	9	58.5	1.44‖	1.45‖
		Avg	1.24 ± .06	1.27 ± .14

* Two animals received each level of casein in both experiments.

† Values in column A are based on Curve A; those in column B are based on Curve B (Fig. 1).

‡ Growth data yielding these values involves consideration of the less reliable lower portion of Curves A and B.

§ Stand. error of the mean.

‖ Calculation of these values involves the less reliable upper portions of Curves A and B.

employed to calculate the tryptophan content of casein from curve B.

The highest values (Table I) calculated from the growth rates with 4 and 9% casein diets appear to result from the use of less reliable portions of the dose-response curves, near or beyond the inflection points. In contrast to these seemingly unreliable values, extremely good agreement between the two experiments was obtained on the 6% and 8% casein diets. All of these values were in the range from 1.22 to 1.33% whether calculated on the basis of percentage of the diet (Column A) or actual tryptophan ingested (Column B). From the most reliable data of Experiment I and all of the results in Exp. II, the best value for the tryptophan content of casein appears to be 1.25%.

Further support for this value is given by amino acid imbalance studies. Experiments in this area have shown that rats develop a niacin deficiency on either a 9% casein diet (13) or purified amino acid diets containing 0.10% to 0.11% of tryptophan and levels of other essential amino acids approaching those required for optimum growth(14). If threonine or another essential amino acid is reduced to a level which is more limiting than tryptophan, the growth rate is improved. It has been found that the tryptophan content of the diet is critical in obtaining such unusual imbalance effects(11). The narrow range, 0.10 to 0.11% of tryptophan or 9% casein in the diet, over which these effects are observed may be used as a basis for roughly calculating the tryptophan content of casein. Thus a value of 1.11 to 1.22% is indicated from the data reported.

For the purposes of comparison with other values in the literature the one arrived at here corresponds to 1.37% when converted to the hypothetical 16% nitrogen basis or to 8.52 mg of tryptophan per 100 mg of casein nitrogen.

Summary. The tryptophan content of casein has been estimated using a rat growth assay method. Whether the results were calculated on the basis of the percentage of tryptophan in the diet or by using food consumption data to calculate the daily intake of tryptophan, essentially the same results were obtained. These animal assays indicate that casein (14.64% N) contains 1.25% tryptophan which is in good agreement with many reports based on microbioassays of alkaline and enzyme hydrolysates of this protein, but much lower than many commonly accepted figures obtained with chemical methods.

The technical assistance of Bonnie J. McLaughlin is gratefully acknowledged.

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Received August 27, 1956. P.S.E.B.M., 1956, v93.

Alteration of Plasma Electrophoretic Patterns and Anaphylactic Reactions in Protein Depleted Dogs.* (22784)

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In 1939 one of us (P.F.H.) reported a means of demonstrating the limitation of hemoglobin production through protein depletion(1). Briefly this method consisted in the forcing of hemoglobin production by maintaining the dog at an anemic level by repeated bleeding, accompanying this by the intravenous administration of colloidal iron in quantities more than equal to the amount of iron removed as hemoglobin, and at the same time maintaining the animal on a diet highly restricted in its protein content. The dog has a propensity to manufacture hemoglobin at the expense of most other body proteins and, as a result, a rapid depletion of protein reserves was achieved. Preliminary studies at the time suggested that the ability of the animal to form antibodies was probably impaired as judged by the fact that the administration of a shocking dose of horse serum 2 weeks after injection of sensitizing doses did not result in a typical anaphylactic reaction. Resumption of the protein depletion work has recently been undertaken with the specific intent to prepare animals which may be suitable for homo-grafts of various tissues. The dog is usually regarded as an unsatisfactory animal for studies of the anaphylactic response, at least insofar as it can be compared with the guinea pig. Nevertheless, we have continued to use this animal for these studies for several reasons, namely: 1. the dog is more amenable to changes in diet than most experimental animals. 2. phlebotomy is considerably facilitated in the dog as compared to small animals and, 3. results of this type of study are much more easily translatable to human studies than in the cases of some of the more widely used small experimental animals. Although skin-graft type of experimentation could probably be equally as well carried out with any of the animals, such studies as survival of homologous kidney transplants are much more practicable in the dog.

We wish to present some preliminary studies relative to depletion of the plasma protein constituents of animals under such a regime. Electrophoretic patterns have been determined on these animals and are now being used as criteria to indicate whether or not the animal has been sufficiently depleted of the protein fractions to warrant an anaphylactic test. The absence or modification of the anaphylactic reaction in such depleted animals is in turn used as an indicator of the animal's readiness for the above-mentioned surgical procedures. Such studies show that, although slight to moderate reduction of the total plasma proteins occurred, marked alteration in the plasma protein makeup was clearly demonstrated.

Methods. Eight litter-mate hounds were used for depletion studies in this series. The animals were adults (over 1 year of age) and weighed from 11.7-13.6 kg initially. They were dewormed, vaccinated against distemper, and maintained on a diet of commercial dog food for 4 months prior to use to assure their status of health and to establish a relatively constant weight, blood picture, etc. During the experimental period they were fed a diet consisting of 300 g of bananas, 100 g of canned salmon, 120 g of Karo syrup, 40 ml of cod liver oil and 1 g of McCollum-Simmonds salt mixture daily. In our early studies the animals maintained or gained weight during the experimental procedure, whereas the animals cited in Table I lost weight throughout the experiment and became quite emaciated. The only obvious difference in the two experimental periods resided in the lowering of the intake of protein during the last 6 weeks of

* Carried out under contract At-(40-1)-269 with Division of Biology and Medicine, USAEC and supported in part by grant from Josiah Macy, Jr. Foundation.

TABLE I. Anaphylaxis in Control Animals.

	Normal	Exp.
Hypernea	8/11	2/8
Salivation	8/11	4/8
Extension	5/11	6/8
Retching	8/11	4/8
Urination	11/11	3/8
Defecation	10/11	3/8
Vomiting	8/11	4/8
Collapse	5/11	1/8
Bleeding tendency	7/11	1/8
Hematuria (gross)	4/11	0/8
Other signs	0/11	1/8
Death	4/11	1/8

the experimental period in our present group, the amount of salmon muscle received in the daily diet having been reduced from 100 to 10 g. It is probable that such an alteration in protein intake may have been quite critical at this stage. Anemia was initially induced by bleeding an estimated ¼ of the blood volume (assuming 72 ml blood per kg) on each of 3 successive days. The animals were then rested for 1 day and the same amount of blood was withdrawn on the 5th day. Subsequent bleeding was such as to maintain an hematocrit value of 20-30%. Iron was administered in the form of the saccharated oxide, "Feojectin"† in quantities which were determined by previous phlebotomy to ensure that an excess existed in stores at all times. No attempt was made to quantitate the amount of hemoglobin removed but rather an estimation was made on a basis of total blood withdrawn and the hematocrit value. The depletion period lasted for 6 months. Electrophoretic studies were carried out on a portable apparatus made by the American Instrument Co., Silver Springs, Md. The plasmas were diluted with an equal volume of diethylbarbiturate buffer of pH 8.6 and an ionic strength of 0.1. The diluted plasmas were dialyzed against the buffer solution for two hours at 1-1.5°C after which the cell was filled. The duration of all runs was 90-120 minutes. Sensitization to the horse serum was accomplished by intravenous administration of 5 ml of horse serum and an additional 5 ml subcutaneously. At the end of 3 weeks 20 ml of horse serum were introduced by vein for anaphylactic test. Following administration of the shocking dose the dog was immediately untied and placed on the floor for observation. Record was kept of subsequent symptoms such as stiffening of legs, excessive salivation, bleeding tendencies, gagging, retching, vomiting, scratching, defecation and collapse. A group of 11 stock animals in apparent healthy condition was sensitized to horse serum at the same time as the test animals, using the same material. When shocking doses of horse serum were administered to this group for comparison with the experimental animals, one dog showed a minimal response inasmuch as there were no symptoms except those of excessive itching as shown by the animal scratching himself vigorously for a prolonged period. The other controls exhibited the usual symptoms such as extension of limbs, retching, collapse, etc. (Table I). Two died within 12 hours. One of the depleted dogs was extremely weak and emaciated at time of the test shock, being barely able to stand alone. Administration of the shocking dose was followed by death without

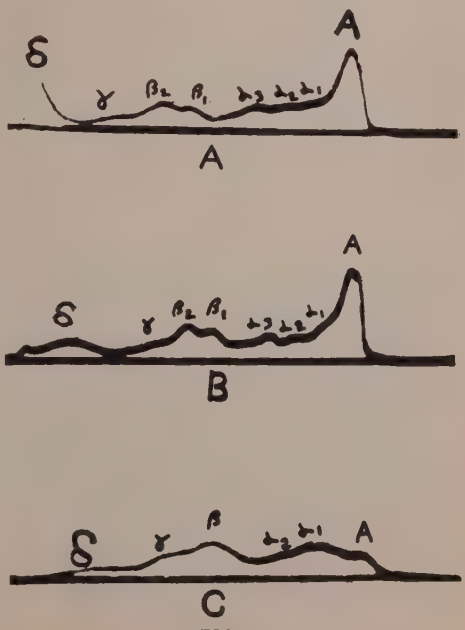

FIG. 1.

† Supplied in generous amounts through the courtesy of Smith, Kline & French Co.

TABLE II. Total Plasma Proteins and Electrophoretic Fractions of Plasma (± S.E.), in 7 Dogs per Series.

Condition of dog	Total proteins*	A/G	Electrophoretic fractions (%)						
			Alb.	Alpha-1	Alpha-2	Alpha-3	Beta-1	Beta-2	Gamma
Normal	6.34 ± .24	.85 ± .05	45.9 ± 1.41	10.3 ± 1.07	7.7 ± 1.16	6.9 ± 1.06	10.4 ± .45	13.3 ± .39	4.8 ± .30
"	5.17 ± .14	.81 ± .09	44.0 ± 2.60	10.7 ± 1.00	9.7 ± 1.37	6.9 ± 1.85	11.7 ± .52	16.3 ± .46	5.0 ± .18
Depleted†	4.86 ± .15	.12 ± .01	10.5 ± .65	29.7 ± .37	12.5 ± 1.19	—	37.7 ± 1.21		9.4 ± .98

* Determined by Semi-Micro-Kjeldahl method.
† Two dogs died during depletion regime.

the common signs of anaphylaxis within a few minutes. In the others the usual manifestations were less severe or absent and they all survived. This phase of the study will be described at length in another communication.

Results. Plasma electrophoretic patterns of the depleted animals are represented by that of dog 54-97 in Fig. 1 which is quite typical of the group.

Table II shows the plasma electrophoretic studies on these 7 litter mate hounds. In each case patterns were run at time of acquisition of the dog and again 5 months later, during which interim the animals were maintained on Purina dog chow and observed closely for abnormalities and for the establishment of baseline hematologic studies. Immediately after the second series of electrophoretic studies the litter was placed on the depletion regime for 6 months. At the end of this time another electrophoretic pattern was determined on each animal. It can be noted that there were only moderate alterations in the *total plasma proteins* with an average reduction of about 20%. This relatively small decrease in total protein content was in contrast to the marked change in the A/G ratio which was decreased from an average of 0.83 under normal conditions to 0.12 in the depleted state or a lowering of approximately 85%. The percentage of albumin ranged from 40-50% in the normal state, while in the depleted state it was reduced to values ranging from 9-13%. In each instance there was a marked increase in total α-globulin. In no case were there 3 alpha globulin peaks found in the dogs following depletion as were found in the normal state. The combined beta-1 and beta-2 globulins were increased consistently in each plasma sample following depletion, the increase amounting to about 1.5 times the value found in the normal state. The gamma globulin values were approximately doubled in the depleted state. This fraction is generally believed to contain most of the antibodies, partial depletion of which by this technic we have reason to feel is accomplished. It is known that the gamma globulin fraction as determined by electrophoresis consists of several components and so obviously a marked decrease in one element with which we are critically concerned may be entirely masked by a concomitant increase in several of the other contributing components. On the other hand it is possible that the antibody with which we are concerned exists in the β_2-globulin fraction.

Summary. 1) Dogs were depleted of body proteins by forced hemoglobin production superimposed on a low protein diet. Electrophoretic patterns indicated markedly decreased albumin values and A/G ratios in spite of relatively little change in total plasma proteins. In all instances there were marked increases in total α, β and γ globulins. 2) Anaphylactic reactions following immunization to horse serum were diminished or absent in the depleted animals as contrasted to controls.

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Received August 27, 1956. P.S.E.B.M., 1956, v93.

Negative Ventricular Diastolic Pressure in Beating Heart Studied *in vitro* and *in vivo*.* (22785)

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Since the classic studies of Harvey(1) it has been generally accepted that diastolic filling of the heart resulted from venous return and auricular contraction. While the senior author(2) was studying the effects of anoxia and work on the glycogen content of the rat heart, it was necessary to make observations on the excised heart beating in saline. With each systole it was observed that fluid ejection from the aorta resulted in the jet propulsion of the heart in the solution and it was obvious that the heart must draw in fluid in each diastole to make possible subsequent systolic ejection; thus it was concluded that part of systolic energy was dissipated in diastole(2).

This paper presents the results of a study of pressure changes in intact and excised heart during systole and diastole.

Materials and methods. White rats weighing 300 to 450 g were anesthetized with pentobarbital 6 mg/100 g. The thorax was rapidly opened and the intact heart removed. A 20 gauge needle was inserted into the left ventricle and the needle connected to a three-way stop cock on a Statham strain gauge so that opening the stop cock recorded hydrostatic zero. The heart was placed in a pottery pie plate filled with saline. The three limb leads of the EKG were placed equidistant around the perimeter of the plate with the electrodes immersed in solution. Pressures and EKG were recorded with an Oscillograph. When desired the auricles were opened widely or excised with a pair of scissors. *In vivo* studies were performed on dogs anesthetized with pentobarbital. Artificial respiration was administered through an intratracheal tube and the chest was widely opened. Umbilical tape was placed around the superior vena cava and azygos veins. In other instances tape was placed around the pulmonary veins. The venous return to right or left ventricle could thus be diminished by tightening the ligatures. A 19 gauge needle with multiple openings in the end was located in the left or right ventricle. The atmospheric zero pressure at the needle tip could be measured by opening stop cock to the air at needle level. The distance between the needle and the highest level of the ventricle was measured to determine the hydrostatic pressure of the column of blood above the needle. The high and low pressures were then charted (Figs. 2 and 3). Electrocardiograms and pressures were recorded on the same record-

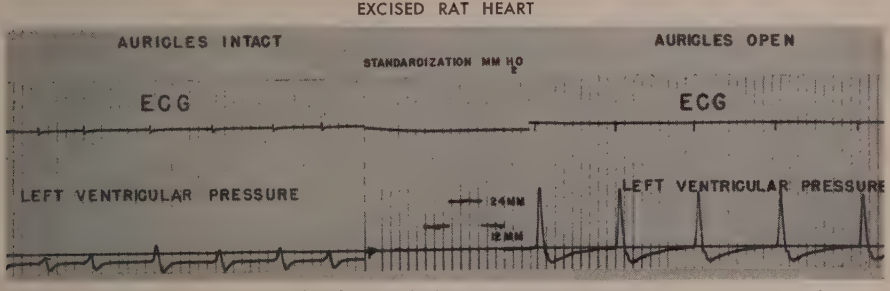

FIG. 1. Electrocardiogram and left ventricular pressure tracing on the excised beating rat heart. The continuous line in the pressure readings indicates hydrostatic zero.

* Presented in part before Meeting of Assn. of Amer. Physicians, May, 1956.

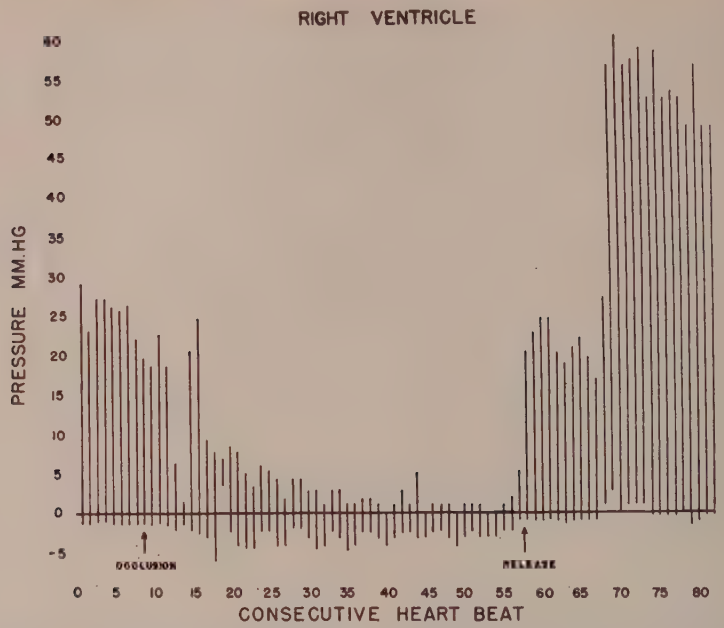

FIG. 2. Highest systolic and lowest diastolic pressure recorded from the right ventricle of the open chest dog. Pressures recorded in respect to atmospheric zero corrected for the hydrostatic column of blood above the needle. Occlusion produced by clamping superior and inferior vena cavae.

ing apparatus used in the rat studies.

Results. Simultaneous electrocardiogram and intraventricular pressure tracings of the excised, beating rat heart suspended in saline are presented in Fig. 1, first with the auricles intact and then with the auricles excised. With the auricles intact the intraventricular pressures were continuously negative, rising toward zero in systole and becoming more negative in diastole. There was no positive intraventricular pressure because there was no filling since the thin walled auricles were collapsed and fluid cannot be drawn through such a freely collapsible structure. With the auricles excised the systolic pressure in the ventricle was positive and the diastolic pressure in the ventricle slightly negative. In this preparation the maximum positive pressure during systole was similar to the maximum negative pressure in diastole with the inflow obstructed. It may be seen that the heart rate slows and the P waves of the EKG disappear when the auricles are excised.

Diastolic pressures recorded in the right ventricle of the intact dog's heart in the open chest were slightly negative and were found to become progressively more negative (–5.0 mm Hg) as the inflow (superior and inferior vena cava, coronary sinus and azygous veins) was occluded (Fig. 2). The systolic pressures also fell (from 27 mm to 2 mm) during this procedure. During each systole a positive pressure developed indicating that venous return was only partially occluded (Fig. 1). Other studies have shown that with further impairment of venous return systolic pressure was further reduced and the shape of the tracings approached that of the excised heart with intact auricles. Following release of venous occlusion the pressures in the right ventricle returned temporarily to pre-occlusion levels.

Left ventricular diastolic pressure was usually 1 to 3 mm Hg below atmospheric pressure (Fig. 3). Diminution of venous return by pulmonary venous occlusion resulted in more negative intraventricular pressures (–15 mm Hg). In other studies pressures as

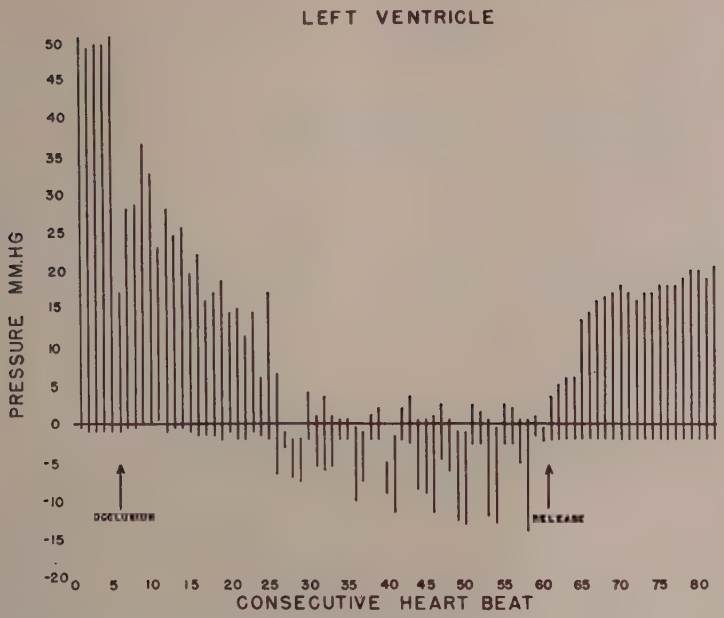

FIG. 3. Highest systolic and lowest diastolic pressure recorded from the left ventricle of the open chest dog. Pressures recorded in respect to atmospheric zero corrected for the hydrostatic column of blood above the needle. Occlusion produced by clamping pulmonary veins.

low as –25 mm Hg have been observed. Systolic pressures also fell (from 50 mm to 3 mm) following impairment of venous return to the ventricle. Following release of venous occlusion the pressures in the left ventricle returned toward normal (from 3 mm to 25 mm) and the diastolic pressures remained negative (–2 mm).

Discussion. The persistence of negative intraventricular pressure during both phases of the cardiac cycle in the excised heart with auricles intact indicated that after the heart empties inflow was impeded by the auricles and fluid cannot be drawn into the ventricles. This results in a continued negative pressure with systole only increasing pressure to zero as a result of contraction. Opening the auricles allows fluid to be drawn through the mitral valve producing a temporary negative diastolic pressure and filling of the ventricle. Systolic contraction of the filled ventricle resulted in a positive intraventricular pressure and systolic ejection of fluid. These pressure changes confirm the observation that the excised heart was capable of drawing fluid into the ventricles during diastole.

The pressure changes in the intact heart of the open chest dog indicated that the *in vitro* findings apply in the intact circulation. The diastolic negative pressures which develop during obstruction of venous return indicated that part of the energy of systole was dissipated during diastole. The left ventricle produced more negative pressures than the right ventricle which might be explained by the differences of thickness and elasticity in the ventricular walls. The slight negativity of pressure in the heart with normal venous return does not indicate absence of a force drawing fluid into the ventricle, but showed that the venous pool was adequate to satisfy the filling force.

The findings suggest that the heart functions as a pump, drawing fluid in during diastole and ejecting fluid in septole. The early rapid diastolic filling observed during cineangiocardiography(3) can be explained by these observations and a mechanism is provided to explain the fact that ventricular volume increases in diastole

when intraventricular pressure decreases(4). Although the concept of the heart drawing fluid in during diastole is old(5,6,7) the magnitude of the negative pressure which can build up in diastole indicates that this mechanism is more important in cardiac filling than has been considered in the past.

We wish to thank Dr. Noble O. Fowler and Dr. E. Ronald Duschesne for their assistance.

Following the preparation of this paper, a communication with Dr. Gerhard Brecher of Department of Physiology, University of Ohio indicates that he has also obtained negative intraventricular pressures in the dog.

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Received August 28, 1956. P.S.E.B.M., 1956, v93.

Effect of Steroid Administration on Excretion of α-Ketolic Steroids and Sulfur in Cats. (22786)

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In the course of treating cats with 9α-fluorohydrocortisone and Δ^1-cortisone (prednisone), the changes in the chromatographic pattern of urinary steroids were measured. Two principal changes from the normal were encountered. (a) The α-ketols which were excreted increased only slightly, but there were changes in the proportions of several steroid metabolites. (b) It was unexpectedly found that elemental sulfur was present in large amounts in the chloroform extracts of these urines and that this interfered with the determination of α-ketols by the blue tetrazolium (B.T.) method, since elemental sulfur was found to give a positive B.T. reaction. After the separation of sulfur and steroids a significant increase in the urinary excretion of sulfur-containing compounds during steroid treatment was observed.

Methods. 9α-Fluorohydrocortisone and Δ^1-cortisone were administered subcutaneously in daily doses of 10 and 50 mg respectively.† The animals were fed diets of fresh meat which were constant during control and treatment periods. After 5 days or more of treatment, all urine was collected for 4 to 7 day periods and pooled. The pooled urine was then incubated with β-glucuronidase at pH 4.5 for 48 hours, extracted with chloroform at pH 1.0, and the chloroform extract washed briefly with 0.1 N NaOH and water. The B.T.-reacting materials were measured as described by Touchstone and Hsu(1). After the determination of total B.T.-reacting substances the remainder of the extract was subjected to paper chromatography.

Results. Fig. 1 shows three representative chromatograms. It appears that the principal metabolites in the normal cat occur in the region where H_4B, allo-H_4B, H_4A and H_4DOC appear (see legend for key to abbreviations). During the administration of 9α-fluorohydrocortisone and Δ^1-cortisone peaks can be found in the regions of H_4F, H_4E, B, A, and H_4DOC. Δ^1-Cortisone also caused excretion of three metabolites which were not found during administration of 9α-fluorohydrocortisone, and it seems that a large portion

* Trainee, National Institute of Arthritis and Metabolic Diseases.

† We are grateful to Merck and Co., and to E. R. Squibb and Sons for supplying these steroids.

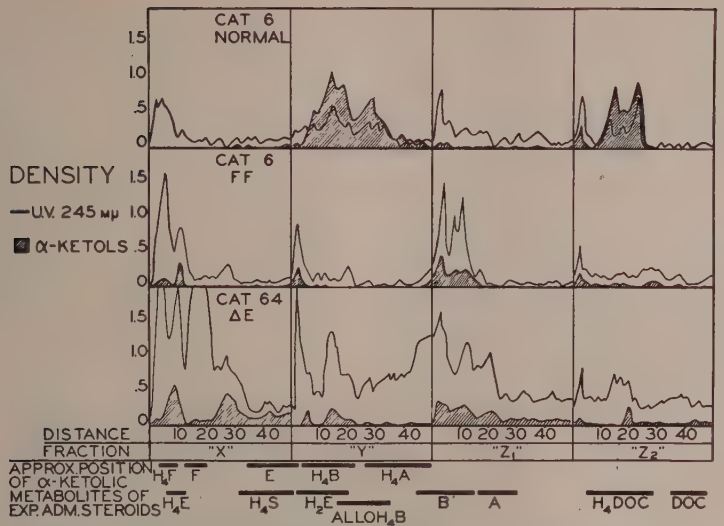

FIG. 1. Representative paper chromatograms of urinary steroid excretion in normal cat and in cats treated with 10 mg of 9α-fluorohydrocortisone (FF) daily and 50 mg of Δ^1-cortisone ($\triangle$E) daily respectively. Chromatograms charted are based on one of eighteen ½ in. strips, each strip representing about 25% of daily steroid excretion(3). Unshaded area represents ultraviolet absorption at 245 mμ, using a Beckman spectrophotometer and special adapter for the chromatographic strips. Steroids having α,β-unsaturated ketone groupings in the A ring will be detected at this wave length, whereas compounds containing a saturated A ring (tetrahydro-forms) show no absorption. The solid silhouette represents α-ketolic compounds which reduce B.T. These were obtained by spraying strips with B.T. and measuring absorption at 600 mμ. No definite identification of the materials represented by these peaks has yet been made. On bottom of chart the approximate locations of the various metabolites in human urines chromatographed under similar conditions by Dohan, Touchstone and Richardson(3) have been drawn. H_4 = tetrahydrocortisone; H_2 = dihydrocortisone; F = hydrocortisone; E = cortisone; S = Reichstein's compound S (11-desoxy-17-hydroxycorticosterone); B = corticosterone; A = 11-dehydrocorticosterone; DOC = 11-desoxycorticosterone. Fraction "X" represents the compounds left on paper after 15-16 cc of effluent/½ in. strip had been collected in a toluene-propylene glycol system. Fraction "Y" represents rechromatography of effluent in the same system, collecting 3 cc of effluent. Fractions "Z_1" and "Z_2" both were chromatographed in methylcyclohexane-propylene glycol, collecting 15 and 5 cc of effluent respectively. Elemental sulfur, but no appreciable amount of α-ketolic steroids, was found in the last effluent "Z_2." Distance—each 10 units = 6.5 cm on paper.

of Δ^1-cortisone is excreted as non-α-ketolic substances. By these patterns, and from histologic sections of the adrenals of the cats(2) it can be inferred that both steroids depress adrenal function.

Determinations on the initial extracts of urine with one application of B.T. yielded surprisingly high values, equalling, or even exceeding, those of man. Erratic results also indicated that the color development was slow and incomplete. By applying B.T. twice, with water washing between applications, higher and more consistent values were found. A third application resulted in no further color development. Cortisone and other steroid standards gave the same results as before. Both the single and double applications yielded a significant increase in B.T.-reacting substances after the administration of Δ^1-cortisone or 9α-fluorohydrocortisone, to the extent that in a few instances the excretion in milligrams exceeded the amount of steroid given. Further investigation showed that the bulk of B.T.-reducing material was elemental sulfur. By the double application method standard solutions of sulfur in chloroform gave a curve conforming to Beer's Law, with a slope similar to that of the steroid standards.

Fig. 2 shows the daily excretion of B.T.-positive materials in cats during control peri-

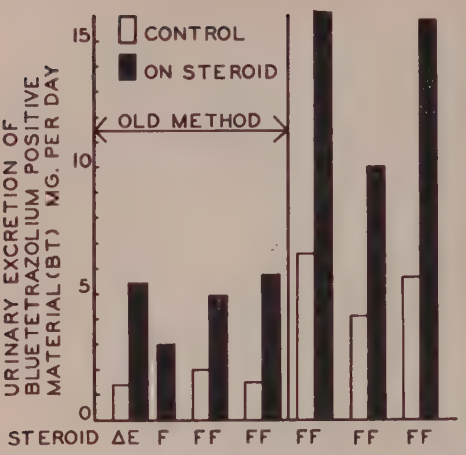

FIG. 2. Daily urinary excretion of blue tetrazolium-positive materials in cats during control periods and during steroid therapy. Old method: One application of blue tetrazolium. The 3 pairs of columns on the right show results after 2 applications of blue tetrazolium. One cat on 10 mg/day of hydrocortisone (F) showed slightly increased excretion. FF = 9α-fluorohydrocortisone.

ods and during steroid therapy. There is a consistent increase during therapy. Not charted are the amounts of B.T.-positive materials in the urine of one Houssay cat and three depancreatized animals, which were all within, or lower than, the normal range.

Fig. 3 shows the relative amounts of sulfur and steroids in extracts from which the sulfur has been separated by chromatography for 12 hours in the methylcyclohexane-propylene glycol system, by which method the less polar sulfur is found in the effluent and the α-ketolic steroids remain on the paper. The amount of steroid is small in comparison to the concentration of sulfur.

Having found that the usual extraction procedure yielded sulfur which interfered with the determination of steroids by B.T., we tried hydrolysis at pH 4.5 instead of pH 1 and found that no sulfur appeared in the extract. Evidently acid hydrolysis is an important determinant in liberating elemental sulfur.

Discussion. These questions now arise: What is the origin of the sulfur, and what is the significance of the increase in sulfur excretion during steroid administration?

Westall(4) reported that cats excrete in the urine large amounts of a peptide, which he called felinine. This compound has been tentatively found to consist of a combination of cysteine and isoamyl alcohol, combined in a thioester linkage. However, extraction of cysteine under the conditions used here failed to show any decomposition to sulfur. The diet does not seem to account for the increase in sulfur excretion, as the cats were fed the same amount of meat before and during therapy.

Negative nitrogen balance *per se*, as demonstrated in the Houssay animal and three depancreatized cats, does not appear to produce an increase in the sulfur excretion. Neither does the presence of glycosuria have an influence, as some steroid treated cats were aglycosuric during the collection period. We have observed that animals treated with steroids have no hair growth, and that the skin is thin and friable. In one cat, gentle handling caused the skin to rupture. There is also severe wasting of muscle, with profound muscle weakness. Both hair, epidermis and muscle are rich in sulfur-containing substances. It is possible that the steroids upset normal metabolism of certain sulfur-containing compounds in these tissues. However, by these methods we have not found sulfur in the urine of man during doses of prednisone up to 80 mg daily.

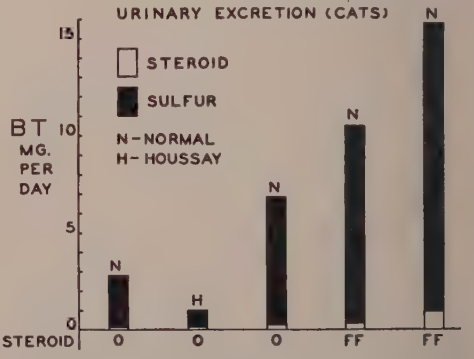

FIG. 3. Daily urinary excretion of blue tetrazolium-positive materials in cats. Sulfur and steroids have been separated. Note that normal untreated cats also excrete sulfur-containing compounds extractable as elemental sulfur. FF = 9α-fluorohydrocortisone.

Summary. 1. Administration of Δ^1-cortisone and 9α-fluorohydrocortisone changes normal urinary excretion of steroid metabolites, indicating adrenal depression or altered steroid metabolism. 2. Only a small proportion of administered 9α-fluorohydrocortisone is excreted as α-ketolic metabolites; Δ^1-cortisone also is converted to a great extent to non-α-ketolic elements. 3. Elemental sulfur has been found to give a positive reaction with blue tetrazolium. This interferes with the quantitative determination of α-ketols in extracts of cat urines. 4. The α-ketols and sulfur can be separated by chromatography and each determined quantitatively by the blue tetrazolium reaction, using a modified method. 5. Administration of Δ^1E and 9α-fluorohydrocortisone causes an increase in urinary excretion of sulfur-containing compounds in cats, which leads us to conclude that these steroids alter sulfur metabolism.

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Received August 28, 1956. P.S.E.B.M., 1956, v93.

Prostatic Neoplasms of the Wistar Rat Induced with 20 - Methylcholanthrene. (22787)

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Prostatic tumors have never been reported to occur spontaneously in the rat, in contrast to man(1) and dog(2,3). McCoy(4) examined nearly 100,000 wild rats finding 103 primary tumors, none of which originated in the prostate. Moore and Melchionna(5) were first to induce with 1:2 benzpyrene in lard squamous cell carcinoma and sarcoma of the anterior lobe of the rat prostate but they did not report metastases. Dunning, Curtis and Segaloff(6) produced metastasizing squamous cell carcinoma in rat prostate with methylcholanthrene but the designation of the prostatic lobe where this carcinoma was induced was not mentioned. Neither groups reported occurrence of adenocarcinoma of the rat prostate.

The purpose of this paper is to report the induction of three types of experimental neoplasms particularly adenocarcinomas in the ventral lobe of the prostate glands of Wistar rats using methylcholanthrene crystals.

Material and methods. 122 male inbred Wistar rats, 3 months of age, were kept in individual cages and given Purina Laboratory chow and water *ad libitum.* Under nembutal or ether anesthesia, the ventral prostate was exposed through a posterior midline incision. Using aseptic technic, a pocket was made in the ventral prostate, and crystals of 20-methylcholanthrene were carefully deposited. The pocket was then closed by suturing with surgical silk. Extreme care was taken to avoid scattering of methylcholanthrene. The technical success of the intraprostatic injections was evidenced by presence of a small yellow bleb. Following the operation, the rats were examined routinely for presence of palpable prostatic masses; biopsies were repeatedly performed to study the character of the growth. This method was used to establish the latent period of the tumor. 23 animals were excluded from evaluation because they died shortly after the operation. The remainder of operated rats, 99 in number, were kept until they showed signs of impending death. These were sacrificed and autopsied. Wherever possible, small fragments of prostatic tumors were removed and immedi-

* This investigation was supported in part by research grants to the senior author from National Cancer Institute, U. S. Public Health Service.

TABLE I. Induced Prostatic Neoplasms in 99 Intact Wistar Rats.

Tumor latent period (days)	Adenocarcinoma	Squamous cell carcinoma	Leiomyosarcoma
100-150		1	
151-200	2	19	
201-250		4	
251-300		2	
301-350		4	3*
Total	2 (2%)	30 (30.3%)	3 (3%)

* 2 rats having leiomyosarcoma had associated squamous cell carcinoma.

ately inoculated subcutaneously into rats of the same strain. The tissue for histologic study was fixed in formaldehyde or Bouin's fluid, embedded in paraffin and sections stained with Harris' hematoxylin and eosin.

Results A. Tumor incidence, latent period and growth behavior of prostatic neoplasms. Table I shows the incidence and latent period of various prostatic neoplasms. Fig. 1 shows a section of a normal mature 6-month-old rat prostate and Fig. 2-5 illustrate prostatic tumors observed in experimental rats. The earliest squamous cell carcinoma was seen on 118th day. Adenocarcinomas appeared on 180th and 198th day. Leiomyosarcoma of

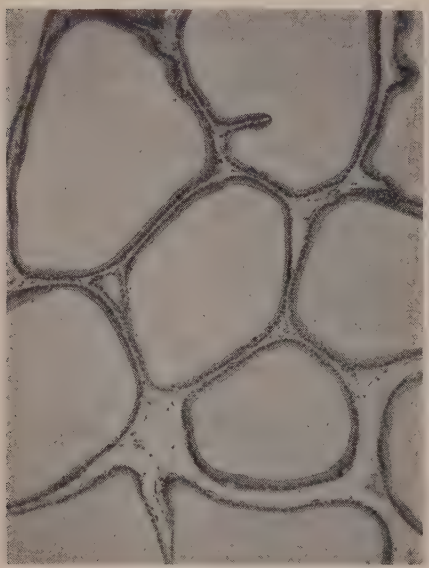

FIG. 1. Section through normal mature 6 mo old rat prostate, ventral lobe. Mag. 150× H & E.

FIG. 2. Photograph of Wistar rat bearing an *in situ* adenocarcinoma of prostate gland. Tumor was 2.8 × 3.2 × 2.5 cm and metastasized to lungs and ribs. Tumor latent period 180 days.

the prostate occurred after 301 days, much later than any of the other neoplasms. In a different series 24 rats that had their ventral prostates sutured only with surgical silk had not developed neoplasms. The average dimensions of the tumors were 3.5 x 4 x 2.5 cm. The squamous cell carcinoma grew the fastest. The average survival time beyond the latent period of rats bearing these tumors was 63 days for squamous cell carcinomas, 92 days for leiomyosarcomas, and 63 days for adenocarcinomas. Death was due to obstruction and/or metastases produced by the tumor. Hydronephrosis, hydroureter, bladder obstruction, anemia and hematuria were complications often seen in rats in which the prostatic masses grew to 3 x 2.5 x 1.5 cm or more. Near death the rats became very irritable, and external and internal bleeding was evident.

Fragments of 21 of the squamous cell carcinomas were transplanted into rats of the same strain. Table III summarizes the growth behavior of 4 of these tumors. Unfortunately, the adenocarcinomas and leiomyosarcomas were not transplanted. Detailed data in Table III on the growth behavior of these transplantable tumors at various generations are advanced to illustrate

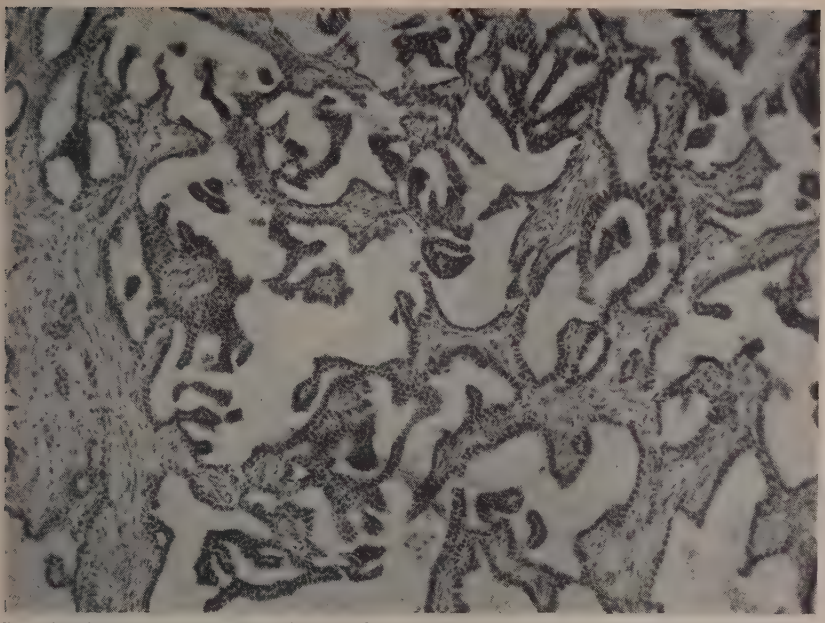

FIG. 3. Section through adenocarcinoma of rat prostate. Sections through tumor mass revealed that there was diffuse infiltration by adenocarcinoma. Carcinomatous acini show characteristic lack of orientation. In some area there was a lack of basement membrane encircling each gland, and irregularity of glandular epithelium. No presence of secretion in the gland was seen. Mag. 150× H & E.

similarities and differences that exist among the 4 tumors. Some of the prostatic tumors that metastasized grew very poorly or not at all in inbred female rats, although the reverse was true when transplanted to male rats.

Histological Findings B. — Of the 35 tumors examined histologically on the basis of cellular structure of the tumor and its staining properties, two were judged to be adenocarcinomas, 30 squamous cell carcinomas, 3 leiomyosarcomas (Fig. 2-5). Invasion and/or metastases of these tumors are summarized in Table II. In most instances, prior to appearance of neoplasms, considerable inflammatory reactions, squamous cell metaplasia and abscess formation were observed, and these reactions were frequently seen in rats that developed squamous cell carcinoma and leiomyosarcoma, but not in rats developing adenocarcinoma of the prostate.

In the 2 adenocarcinomas examined, the tumor cells were arranged in glandular formation around irregular spaces, or trabeculae and the stroma was composed of a small

TABLE II. Frequency Distribution of Metastases and/or Invasion of Induced Prostatic Neoplasms in Wistar Rats. Leiomyosarcoma did not metastasize.

Organ	Squamous cell carcinoma	Adenocarcinoma
Lung	10	1
Thymus	1	0
Heart	1	0
Liver	3	0
Spleen	1	0
Pancreas	1	0
Adrenal	3	0
Kidney	4	0
Lymph nodes	8	0
Intestines	5	0
Seminal vesicle	12	0
Coagulating gland	12	0
Dorsal prostate	14	0
Testes	2	0
Epididymis	6	0
Ribs	2	1
Pelvic bone	0	0
Bladder	11	1
Diaphragm	3	0

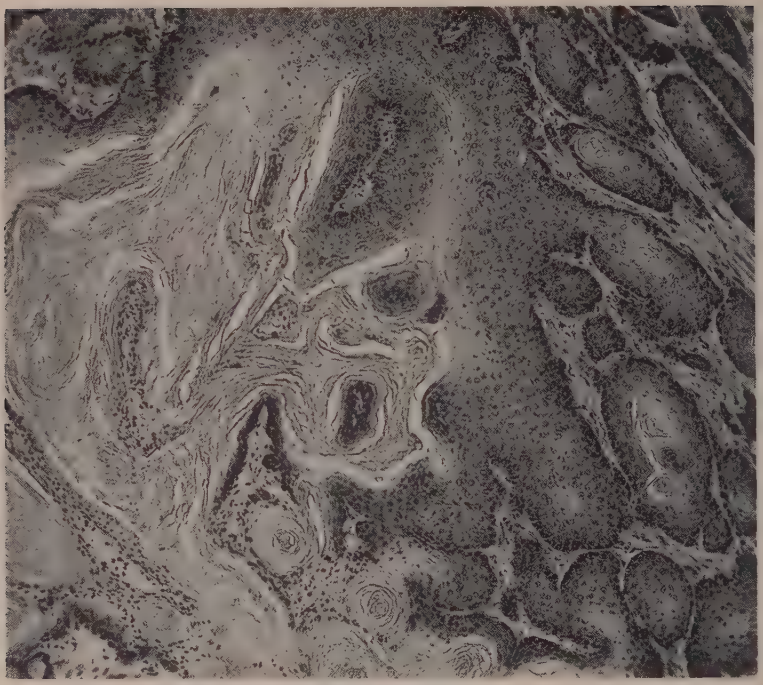

FIG. 4. Section through squamous cell carcinoma of rat prostate. Tumor measured was 3.9 × 4.8 × 2.2 cm and metastasized to lung, rib, kidney, mesentery, and omentum. Mag. 150× H & E.

amount of connective tissue accompanied by slender capillaries. The tumor cells had round or oval hyperchromatic nuclei of varying size situated in the basal part of the cells. The cytoplasm appeared more swollen and foamy or vacuolated and the nucleus became pyknotic. No evidence of secretion was seen in the gland with diffuse infiltration by adenocarcinoma.

The leiomyosarcomas consisted of cells that were large and spindle-shaped with abundant eosinophilic cytoplasm; among these were many giant cells. In some areas of the tumors, the cells showed pleomorphism with round or oblong nuclei as the predominant form and many mitotic figures were common. The cells of this tumor often tended to form interlacing bundle running in different directions. Two of the three rats with leiomyosarcomas that had a latent period of 301 and 308 days respectively had associated with it areas of squamous cell carcinoma. Although the leiomyosarcomas did not metastasize, the general histological appearance supported the belief that the tumors were malignant.

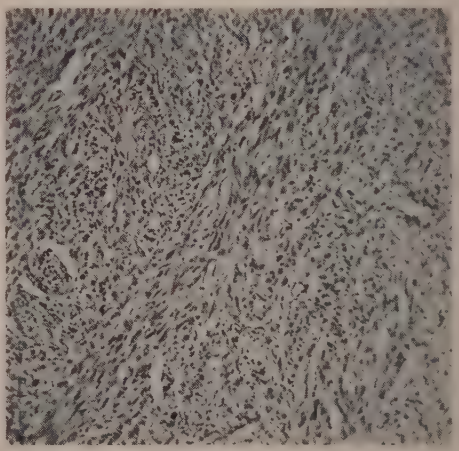

FIG. 5. Section through rat prostate with leiomyosarcoma. Tumor measured 2.8 × 2.2 × 3.0 cm, grew slowly and did not metastasize. Glandular nature of prostate was completely absent. Mag. 125× H & E.

TABLE III. Results of Homologous Transplantation of Several Prostatic Squamous Cell Carcinomas into Wistar Rats.

Designation No. of tumor	Response of tumor at generation	No. of rats transplanted into	Growth characteristics: Site in host inoculated	Takes, %	Latent periods, days	Kill host, days	Infiltration and metastases	Special features
1	12	12 ♂	Subcut.	90	8–10	30–40	Local invasion, lung	No regression, ulceration
2	18	12 ♂	"	60	14–21	30–60	Local invasion	Regression after ulceration (15%)
		12 ♀	"	70	12–18	25–52	*Idem*	" " (20%)
3	16	12 ♂	"	85	7–14	25–30	Lung, lymphnodes	Hemorrhage, necrosis, no regression
		12 ♀	"	20	14–22	40–56		Hemorrhage, necrosis, regression
		12 ♂	Intraper.	56		44–54	Lung, ribs, local invasion	Necrosis, hemorrhage, no regression
		12 ♀	"	0				
		12 ♂	Pleura cavity	36		18–23	Lungs, ribs, adrenal diaphragm	Necrosis (less extensive), hemorrhage, no regression
		12 ♀	*Idem*	0				
4	10	12 ♂	Subcut.	25	14–28	50–60	Lung, occasional local invasion	Regression rare, necrosis (less extensive)
		12 ♀	"	10	24–28			Regression

Histologically most squamous cell carcinomas were composed of irregular masses or sheets of more or less differentiated basal and columnar cells with an inconspicuous stroma. In many of the tumors, the cancerous cells formed epithelial pearls or small cystic areas with mucoid material in the center. In some tumors the epithelial elements which differentiated and formed keratin, also had areas that appeared glandular. It seemed as if the "neoplastic" change did not go on to full squamous cell differentiation.

Discussion. It has been shown that prostatic neoplasms, similar to the 3 types that occur in man, can be induced with methylcholanthrene in the ventral prostate of Wistar rats, squamous cell carcinoma being more frequent in the rat than in man. Adenocarcinoma of the prostate gland in the rat showed great similarity to that of man. Moreover, these neoplasms with the exception of leiomyosarcoma not only exhibited extensive local growth and invasion, but also distant metastases. Judging by distribution of metastases in various organs, the tumors were autonomous, and spread was through blood and/or lymphatic pathways.

The fact that this non-specific, poorly soluble carcinogen induced so high a proportion of prostatic squamous cell carcinomas proved not to be out of keeping with the results of Moore *et al.*(5) and Dunning *et al.*(6). On the other hand, our results differ from the above investigators by the induction of adenocarcinomas and leiomyosarcomas, by a more extensive distribution of invasion and/or metastases of prostatic tumors to various organs, and by a more detailed account on the growth characteristics of transplantable prostatic tumors, some of which are in their 18th transplantable generation. Moore *et al.* and Dunning *et al.* used a different strain or subline of rats in their respective studies. It is noteworthy to point out that different species and sometimes even different strains of the same species vary markedly in the relative sensitiveness of their various tissues to induced and spontaneous tumor development. Moreover, the nutritional, hormonal and metabolic status of the gland are important

factors too, that can greatly affect the tumorgenic response of a gland to a carcinogen.

It is becoming increasingly important that when working with the prostatic gland of the rat that the designation of the prostatic lobes be carefully indicated despite having a common origin arising as buds of the urogenital sinus. The observation that the prostatic lobes in an animal is not a homogenous structure as judged by its response to estrogen, enzyme concentration, chemical constituents (7) and zinc^{65} uptake(8,9) has been late in discovery notwithstanding the fact that histological procedures do not reveal any differences among the lobes of the prostate gland. It is therefore reasonable to assume that lobes of the rat prostate gland can react differently to methylcholanthrene or to other carcinogens. Preliminary studies using different carcinogens to various prostatic lobes seem to support this concept.

Summary. (1) Intraprostatic deposits of 20-methylcholanthrene crystals induced three types of prostatic neoplasms in the ventral lobes of the Wistar rat's prostate gland: adenocarcinoma (2%), leiomyosarcomas (3%) and squamous cell carcinomas (30.3%). The squamous cell carcinomas and adenocarcinomas metastasized. (2) A detailed account of the distribution of invasion and/or metastases of these prostatic tumors to various organs are presented as well as an account on the growth characteristics of transplantable squamous cell carcinomas.

The authors wish to express their thanks to Miss Helen Fox, Edward Dywinski, and Andre Bulba for technical assistance, Dr. L. Simpson for her assistance in diagnoses of histological slides.

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Received August 30, 1956. P.S.E.B.M., 1956, v93.

Nitrogen Sparing Action of Neutral Fat and Fatty Acids in the Phlorhizinized Rat.* (22788)

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In a recent review devoted to the relationship of protein metabolism to that of fat and carbohydrate, Munro summarized the extensive data indicating that carbohydrate is a protein sparer, regardless of whether the animal is starving or is receiving a protein free or protein containing diet(1). By contrast, Munro noted that the results of studies dealing with the ability of fat to prevent excessive nitrogen loss are in conflict. Thus, some reports indicate that when fat is the sole source of calories, the nitrogen output of an animal is as great as during total starvation(2,3,4,5); on the other hand, data has been presented which demonstrates that fat is a protein sparer, even in the absence of protein in the diet, if minimal caloric requirements are satisfied(6,7). In view of the divergent results, and because it seemed to us that fat, under

* This investigation was supported by research grant from National Institute of Arthritis and Metabolic Diseases, Public Health Service.

† This department is supported in part by the Michael Reese Research Foundation.

TABLE I. Composition of Diets.

Vitamins and salt			
Salt (Wesson)	20 g	Vit. B syrup	30 ml
Cod liver oil	5 ml	2-methyl naphtho-quinone	50 mg
Wheat germ oil	5 "		

To the above were added, for the respective diets:

	High carbohydrate	Moderate carbohydrate	High fat
	g		
Casein hydrolysate (enzymatic)	95	95	95
Lactalbumin hydrolysate (enzymatic)	10	10	10
Methionine	2	2	2
Liver powder	10	10	10
Corn starch	185	110	
Dextrin	170	105	
Sucrose	170	110	
Mazola oil	10	100	245
Gum ghatti			10

Solid diet: To moderate carbohydrate diet were added 300 ml of 5% agar agar and 300 ml of water.

Liquid diets: To appropriate diet were added 30 g of cellu flour and water to make 1000 ml.

Protein-free diets: Made up similar to liquid diets with exception that casein and lactalbumin hydrolysates, methionine and liver powder were omitted.

Oleic acid diet: To vitamins and salt, 30 g of cellu flour, 10 g of gum ghatti, 240 g of oleic acid and water to make 1000 ml were added.

Glycerol diet: To vitamins and salt, 30 g of cellu flour, 10 g of gum ghatti, 25 g of glycerol and water to make 1000 ml were added.

appropriate conditions, should spare protein in the phlorhizinized rat, the experiments described below were undertaken. Protein sparing effects were obtained in phlorhizinized rats tube fed protein free diets, irrespective of whether the calories were supplied by neutral fat, fatty acids or carbohydrate.

Methods. Male Holtzman rats, weighing approximately 120 g at the start of the experiment, were employed and fed diets modified from those described by Ingle(8). The food was available to the animals eating *ad libitum* in solid form at all times but was administered by stomach tube to the force fed groups in liquid state twice a day, before 9 a.m. and after 4 p.m. The compositions of the diets are given in Table I. Four series of rats were employed: (1) Group 1 was *ad libitum* fed the solid moderate carbohydrate diet; (2) Group 2 was force fed the moderate carbohydrate diet; (3) Group 3 was force fed the high carbohydrate diet; (4) Group 4 was force fed the high fat diet. The rats were placed on their respective diets for 3-4 weeks, until they weighed between 210 and 220 g, the amount of diet administered to the rats in the force fed series being adjusted so that the weight gains of these animals approximated that of the *ad libitum* fed ones. At the completion of the feeding period, the rats were injected intramuscularly on each of 2 successive days with 50 mg of phlorhizin in 1 ml of sesame oil. The dietary management of the animals during the 2 days is shown in Table II. All animals were placed in individual metabolism cages during the second day of phlorhizinization and their urines collected under toluene with a gram of citric acid as an acidifying agent. These 24 hour specimens

TABLE II. Dietary Management of the Rats during the Two Days of Phlorhizinization.

Rat group	Period of starvation		Protein-free diets fed*				
	Day 1	Day 2	Moderate carbohydrate	High carbohydrate	High fat	Oleic acid	Glycerol
1†	+	+	0	0	0	0	0
2‡ (a)	0	0	+	0	0	0	0
(b)	+	0	+	0	0	0	0
3§	0	0	0	+	0	0	0
4‖ (a)	0	0	0	0	+	0	0
(b)	+	0	0	0	+	0	0
(c)	+	0	0	0	0	+	0
(d)	+	0	0	0	0	0	+

* Diet force fed the day(s) the animals were not starved. † These rats previously were on *ad lib* diet. ‡ Had been previously force fed the moderate carbohydrate diet. § High carbohydrate diet. ‖ High fat diet.
+ = Treatment indicated; 0 = no treatment given.

TABLE III. Excretion of Nitrogen and Glucose after Injections of Phlorhizin.

Rat group†		N excreted* mg/100 g body wt	Glucose excreted* mg/100 g body wt
1‡	(7)	125 109–156	331 233– 429
2§ (a)	(6)	42 39– 54	946 743–1220
3‖	(7)	35 28– 41	1053 790–1305
4¶ (a)	(5)	67 54– 88	445 413– 500

* Values given are mean excretions and ranges for the group.

† No. in parentheses refers to No. of animals in group.

‡ These rats had previously been fed *ad lib*; they were starved for 2 days of phlorhizinization.

§ These rats were force fed the moderate carbohydrate protein free diet for 2 days of phlorhizinization.

‖ Force fed high carbohydrate protein free diet for 2 days of phlorhizinization.

¶ Force fed high fat protein free diet for 2 days of phlorhizinization.

TABLE IV. Excretion of Nitrogen and Glucose after Injections of Phlorhizin.

Rat group†		N excreted* mg/100 g body wt	Glucose excreted* mg/100 g body wt
1‡	(7)	125 109–156	331 233– 429
2§ (b)	(5)	40 37– 43	1126 803–1394
4‖ (b)	(6)	59 43– 75	463 176– 947
(c)	(5)	62 55– 81	368 211– 424
(d)	(6)	47 38– 57	482 404– 634

* Values given are mean excretions and ranges for the group.

† No. in parentheses refers to No. of animals in group.

‡ These rats had previously been fed *ad lib*; they were starved for 2 days of phlorhizinization.

§ These rats were starved for the first and force fed the moderate carbohydrate protein free diet for second day of phlorhizinization.

‖ These rats were starved for first day of phlorhizinization; on second day, rats of groups (b), (c) and (d) were force fed protein free high fat, oleic acid and glycerol diets respectively.

were analyzed for N.P.N. by a modified Conway technic(9) and for reducing substances by the method of Nelson(10).

Results. Table III shows the urinary outputs of N.P.N. and glucose for the second day in rats force fed protein free diets during the 2 days of phlorhizinization, as well as the urinary losses of the starving animals. It can be seen that the N.P.N. output dropped from 125 mg/100 g in the animals who did not eat to approximately 40 mg/100 g in the animals force fed the carbohydrate diets. Little difference can be noted between the nitrogen excretions of the animals receiving the high or moderate carbohydrate intakes. When fat in the form of mazola oil was the source of calories, the nitrogen loss fell to approximately 67 mg/100 g of rat.

Starvation during the first day of phlorhizinization, followed by the feeding of the respective protein free diets on the second day, resulted in outputs similar to those noted above (Table IV). Fat feeding, in the absence of protein or carbohydrate, appeared to decrease the urinary nitrogen excretions.

The D:N ratios obtained in the above experiments suggested that gluconeogenesis from fat was occurring. However, since the dietary source of fat (mazola oil) is at least 90% trioleate‡ and therefore contains 10% available glycerol when hydrolyzed, it became apparent that both the "gluconeogenesis" and protein sparing we were observing might be attributable to the glycerol moiety of the fat. Accordingly, two groups of rats who had been force fed the high fat diet for 3-4 weeks were phlorhizinized for two days, starved on the first day but on the second day were force fed oleic acid or glycerol in the amounts calculated to be present in the high fat (mazola oil) diet. From the results in Table IV, it may be seen that the oleic acid, in the absence of glycerol or any other form of carbohydrate, prevented the excessive protein catabolism that results from phlorhizinization.

Discussion. The results obtained in our phlorhizinized rats, fed isocaloric amounts of protein free diets containing large or moderate amounts of carbohydrate or large amounts of neutral fat or fatty acids indicate that fat can inhibit endogenous protein catabolism. The

‡ We are indebted to Corn Products Refining Co. for this information.

degree to which fat prevents protein breakdown was not so great as that observed with carbohydrate; with the latter diets, N.P.N. excretions were decreased by 68%, with mazola oil by 53% and with oleic acid by 50%. Glycerol, in amounts of only 500 mg per rat per day decreased N.P.N. outputs by 63%.

The fats usually employed in laboratory diets are essentially triglycerides and contain 10% glycerol in ester linkage. In a high fat diet, as much as 500 mg/day of glycerol may come from this source. Failure to appreciate that 10% of dietary fat becomes available as carbohydrate when metabolized may be a factor in explaining the conflicting results obtained in previous studies.

Our first experiments were performed on animals force fed their respective protein free diets during the 2 days of phlorhizinization. To rule out the possibility that the apparent sparing of protein by fat may have been associated with the force feeding on the first day of phlorhizinization, thereby conserving body carbohydrate and protein stores, observations were made on animals starved for the first day of phlorhizin injections. On the second day, the animals were given their respective protein free diets or the oleic acid or glycerol diets (Table II). As is evident from Table IV, carbohydrate, neutral fat or fatty acids still prevented the usual protein loss seen with phlorhizinization.

While our findings are in accord with previously published data on the nitrogen sparing action resulting from feeding carbohydrate to phlorhizinized animals, they do not agree in respect to fat feeding. It has been reported that when fat, in the absence of protein, is administered to phlorhizinized animals, the accelerated nitrogen loss continued unabated (11,12,13). In contrast to previous work, we find a marked decrease in N.P.N. excretion when a protein free diet containing neutral fat or fatty acids is administered. The difference between our results and those of others may possibly be attributed to the preexperimental dietary history of the animals; our rats were adapted and maintained on a high fat diet for 3-4 weeks before they were phlorhizinized whereas previous workers did not so adapt their animals. Munro is of the opinion that for the nitrogen sparing action of fat to be seen, studies should be performed several days after the animals have been placed on the high fat regimen(14).

It is not surprising that fat, like carbohydrate, should be found to spare nitrogen. Protein, carbohydrate and fat are considered to share a common final metabolic pathway with respect to carbon dioxide production (and energy yielding reactions). When there is a demand for energy, carbohydrate is probably utilized preferentially to protein. The findings in the carbohydrate fed phlorhizinized rats are in accord with this reasoning. On the basis of our results in the animals fed fatty acids during phlorhizinization, it appears that fat likewise is utilized in preference to endogenous protein for energy yielding reactions. However, since fat is not thought to be converted to carbohydrate, the use of protein for carbohydrate formation should cause fat fed phlorhizinized animals to excrete more nitrogen than carbohydrate fed ones. Our results are in agreement with this possibility since the N.P.N. excretions of the animals fed fat were slightly greater than the excretions of the ones fed carbohydrate.

Summary. Rats were adapted to the force feeding of a moderate or high carbohydrate or high fat diet for several weeks before phlorhizinization. During the second day of phlorhizin injections, the animals were fed their respective diets, rendered protein free, and the urinary excretions of N.P.N. measured and compared to the outputs of starving animals. The feeding of either neutral fat or carbohydrate reduced N.P.N. losses. A similar sparing of endogenous protein occurred when animals, fed the high fat diet for 3 weeks, were given oleic acid, rather than mazola oil, on the second day of phlorhizinization.

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Received August 31, 1956. P.S.E.B.M., 1956, v93.

Ergotamine, Oxytocin and Milk Let-Down in Lactating Rat.* (22789)

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It has been demonstrated(1-4) that adrenalin in appropriate doses can block the milk let-down response to both endogenous and injected oxytocin. Adrenalin presumably acts by constricting mammary blood vessels thus blocking passage of oxytocin to mammary myoepithelium(5-6). Ergotamine, also a peripheral vasoconstrictor(7), has recently been shown to inhibit milk let-down in lactating rats(8). The present paper is concerned with effects of exogenous oxytocin on ergotamine-inhibition of milk let-down in lactating rats. Evidence is presented to indicate that ergotamine exerts its inhibition by means other than through vasoconstrictive effects.

Materials and methods. Docile lactating albino rats, each with its first litter and weighing 200-260 g, were housed in individual cages and given free access to food and water. Each litter was reduced to 8 young shortly after birth and, whenever possible, these were equally distributed as to sex. Rats with less than 8 young were discarded. Each of 42 litters when 14 days old was isolated from their mother for 10 hours. During this time they were kept warm to prevent any undesirable effects from exposure. Each litter was then divided equally. One half was weighed as a group, killed and their stomachs examined for the presence of milk. The other half was replaced with the mother and allowed to suck for exactly 20 minutes. Length of time before each mother began nursing was recorded. Young were then removed, weighed as a group and immediately killed by decapitation. Their stomachs were opened and semisolid curds removed and weighed (Table I). The mothers received single subcutaneous injections as follows: (a) 17 received 1 mg/kg ergotamine tartrate.‡ Of these 10 were injected 10 minutes, the rest, 2 minutes before replacement of litters. (b) 10 received 1 mg/kg ergotamine 10 minutes before replacement of litters followed by oxytocin ('Pitocin', Parke Davis & Co.) 8 minutes later. Oxytocin was given to 5 rats in dose of .4 I.U./kg and to remaining 5 in dose of .2 I.U./kg. (c) 5 were treated with .2 I.U./kg oxytocin 10 minutes before replacement of litters followed by 1 mg/kg ergotamine 8 minutes later. (d) 10 served as controls of which 5 were uninjected. The remaining received comparable injections of distilled water 10 minutes prior to replacement of litters. Topical effects of saline solutions of 1:2000 ergotamine and .2 I.U./ml oxytocin alone, and in combination, were studied in 5 lactating rats. Each mother was separated 4-6 hours from its litter on the 14th day postpartum. Nembutal anaesthesia (3 mg/

* Contribution from Department of Dairy Husbandry, Mo. Agr. Exp. Station. Journal Series No. 1664. Approved by Director.

† Postdoctoral Fellow of the National Institutes of Health.

‡ Kindly supplied by Sandoz Chemicals, N. Y.

TABLE I. Average Quantity of Milk, Expressed as % Litter Body Weight, Secured in 20 Min. by Litters, Each with 4 Young, on the 14th Day Postpartum.

Treatment	No. of rats	Time of treatment prior to nursing (min.)	Avg wt of litters (g)	Avg wt of milk (g)	% milk / Litter wt	S.E.
Control	10	10	89.2	3.3	3.7	.43
Ergotamine, 1 mg/kg	10	10	86.8	.6	.7	.21
Idem	7	2	94.0	2.0	2.1	.68
Ergotamine, 1 mg/kg, then oxytocin, .2-.4 IU/kg	10	10 2	96.9	4.2	4.3	.31
Oxytocin, .2 IU/kg, then ergotamine, 1 mg/kg	5	10 2	97.7	4.2	4.3	.51

100 g) was injected intraperitoneally, an abdominal mammary gland exposed and observed with a stereoscopic dissecting microscope (x16-x32). The exposed tissue was kept moist with physiological saline solution and covered with a thin sheet of polyethylene. Drugs were administered as drops at the tip of a 24 gauge needle and gently placed on mammary tissue without touching it with the needle.

Results. At the end of 10 hours isolation, stomachs of half of each litter contained no milk. It was assumed, therefore, that stomachs of other half of each litter permitted to suck following isolation, were also empty prior to sucking. In each case upon placing the litter back with their mother after isolation, she was observed to hastily gather her young and to commence nursing within 3 minutes.

Amount of milk obtained by litters of lactating rats injected with ergotamine 10 minutes prior to litter replacement was very small in comparison with that obtained by control offspring ($P = .001$), or with that obtained by young of mothers treated with ergotamine 2 minutes before litter replacement ($P = .03$). This latter group obtained an average of only 57% as much as control offspring though the quantity difference was not significant ($P = .06$). When oxytocin was injected before or after ergotamine insignificantly larger ($P = .4$) quantities of milk were obtained by offspring in comparison with control group. The amount was, however, significantly greater than that obtained by litters of rats treated with ergotamine either 10 or 2 minutes prior to replacement of young. ($P = .001$ and $.005$ respectively). There was little difference in amount of milk obtained by offspring of uninjected and distilled water-injected controls.

Topical administration of ergotamine to living rat mammary tissue resulted in constriction of arterioles but not of venules or of alveolar capillaries. Constriction occurred within 1-10 minutes with relaxation taking place 3-15 minutes later, depending upon vessel diameter. Effect on mammary alveoli and ducts varied from no response, in most instances, to slight transient contraction of a few alveoli. Oxytocin administered alone caused almost immediate (2-4 sec.) contraction of alveoli which resulted in ducts filling with milk but there was no apparent effect on the vascular bed. If the glands were not engorged, milk did not return to the alveoli, which remained contracted. If alveoli were greatly distended, milk squeezed into ducts gradually ran back into alveoli again. Such relaxation occurred within 1-5 minutes. It was possible, therefore, in some cases to apply ergotamine and/or oxytocin to same area of tissue and obtain another contractile response as soon as relaxation of alveoli and arterioles occurred.

Ergotamine and oxytocin mixed together and applied topically, evoked both alveolar contraction and arteriole constriction. Moreover, oxytocin applied at height of ergotamine-induced arteriole constriction, elicited its usual alveolar contraction with no apparent delay in contraction time. Drops of saline applied directly to mammary tissue had no apparent effects.

Discussion. These observations would

seem to rule out ergotamine inhibiting milk let-down in lactating rats by vasoconstriction of mammary vessels. There remain at least two other possibilities to explain this inhibition. It is generally accepted(9,10) that mammary myoepithelial cells are the contractile elements responsible for squeezing milk from alveoli and ducts. There may then be a competition between oxytocin and ergotamine at the myoepithelium level for results from injecting ergotamine 2 minutes prior to nursing, suggests a certain level of the drug must be present in general circulation before full inhibition of milk let-down is assured. The other possibility is ergotamine through sympatholytic action and central nervous system depression blocks release of oxytocin from posterior pituitary gland. As neural pathways involved in the milk let-down reflex are obscure, it is perhaps premature to speculate on the site of neural blockage by ergotamine. It has been shown, however, that electrical stimulation of anterior hypothalamus(11) and vagi(12) in lactating sheep and goats, and supraoptico-hypophyseal tract(2,13,14) in lactating rabbits resulted in let-down in milk. It is possible that ergotamine inhibits milk let-down in lactating rats through blockage of one or more of these areas.

Summary. 1. The quantity of milk secured by litters of lactating rats treated with ergotamine 10 minutes before nursing was significantly less than that obtained by control offspring. Oxytocin administered before or after ergotamine restored normal milk let-down. 2. Topical application of ergotamine to living rat mammary tissue resulted in arteriole constriction but did not interfere with normal myoepithelial contraction induced by oxytocin. 3. Results indicate ergotamine does not inhibit milk let-down through vasoconstriction of mammary blood vessels thus prohibiting access of oxytocin to effector myoepithelium. Rather, a competition between ergotamine and oxytocin for the effector tissue or a neural block inhibiting release of oxytocin by posterior pituitary gland seem more probable.

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Received September 4, 1956. P.S.E.B.M., 1956, v93.

Fixation of Carbon Dioxide by *Actinomyces* and *Lactobacillus bifidus*. (22790)

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In recent studies on fermentation of glucose by *Actinomyces bovis* and *Lactobacillus bifidus*(1) a net fixation of CO_2 was definitely observed in some fermentations and suggested in others. The overall stoichiometry of 2 fermentations by *Actinomyces* indicated that CO_2 was being fixed in a manner consistent with the operation of the Wood-Werkman reaction(2) with the formation of succinic acid. The data suggested that this same reaction was functioning to a minor extent in one strain of *L. bifidus;* but that the major path-

way in this organism was one which satisfied the equation: glucose→3 acetic acid. In view of the facts that the same stoichiometry of glucose fermentation occurs in fermentations by *Clostridium thermoaceticum*(3), that carbon dioxide is reduced to acetic acid in the clostridial fermentation(4,5), and that both *Actinomyces* and *Lactobacillus bifidus* have been shown to require carbon dioxide for growth under certain conditions(1), it was decided to conduct fermentations in the presence of carbon dioxide-C^{14} and to determine the extent of incorporation of radioactivity into the carbon atoms of the products.

Materials and methods. The organisms used were *Actinomyces bovis*, strains ATCC #10048 and #10049, and *Lactobacillus bifidus*, strain #308, obtained from Dr. Paul Gyorgi, University of Pennsylvania Hospital. They were grown on casitone-starch medium with 1% glucose in fermentation tubes as described earlier(1). However, sodium carbonate-C^{14} instead of sodium carbonate-C^{12} was used in the pyrogallol seal to induce anaerobiosis. In aerobic fermentation described in Table II, the culture grew anaerobically 24 hours. The culture was then attached through the top stopper of fermentation tube to a one liter flask containing pure oxygen, and the culture mixed for additional 24 hours with magnetic stirrer. Quantitative determinations of the products were done as described previously(1). Pyruvic acid was identified and estimated by the method of Koepsell and Sharpe(6). Formate was degraded in the presence of acetic acid by oxidation with mercuric sulfate and recovered and counted as barium carbonate. The residual acetic acid was steam distilled and its purity verified by its Duclaux constant. In Table I, acetate was degraded by wet oxidation to determine total specific activity; radioactivity in the carboxyl carbon was determined using the Schmidt degradation(7); and radioactivity of methyl carbon was calculated as the difference. In all other fermentations acetate was recovered as barium acetate and counted as such, applying self absorption corrections determined for barium carbonate. In the fermentation in Table I, succinic acid was separated from lactic acid using a celite column according to procedure of Phares *et al.* (8). Only those fractions were used which gave a negative spot test for lactic acid(9). The succinate was degraded as described below. In all other fermentations, non-volatile acids were chromatogramed on paper (S and S #507) using ammonia-ethanol-water solvent of Cheftel *et al.*(10). Acid bands were demonstrated using brom cresol green (0.05%) adjusted to blue with 0.1 N NaOH. A much more sensitive and satisfactory spray was 0.3% solution of ninhydrin used after the papers were air dried and no longer smelled of ammonia. The color was developed in a 105°C oven for 5 to 10 minutes. This procedure was used in those cases where the acids were not recovered from the paper. The radioactive bands obtained from the *Actinomyces* fermentations were located by radioautograms and were checked for coincidence with the acid bands. The acids were identified by their Rf values and were cut from the paper using the radioautogram as a guide. The single radioactive band obtained from the *L. bifidus* fermentation was located using a hand probe. In all cases there was a wide separation of the lactic acid band from that of the succinic acid. In all cases radioactivity and acid bands were exactly coincident

TABLE I. Anaerobic Fermentation of Glucose plus Carbon Dioxide-C^{14} by *Actinomyces* Strain #10049.*

Substrate or product	μmoles/ml	Cts/min. /μmole
Glucose utilized	34.4	
Carbon dioxide (initial)	33.4	523.4
" " (final)	14.1	492.8
Formic acid	12.6	50.6
Acetic acid	10.3	.36
–COOH		.23
$-CH_3$		.13
Lactic acid	43.2	n.d.†
Succinic acid	11.0	527.2
–COOH		143.3
$-CH_2-$		120.3
Beta alanine (predicted)		383.9
(found)		377.0

* Fermentation of glucose was done by the fermentation procedure described by Pine and Howell (1) using a 1% glucose casitone medium with starch.

† n.d. = not determined.

TABLE II. Fermentations of Glucose plus Carbon Dioxide-C^{14} by *Actinomyces* Strains #10048 and #10049 and by *Lactobacillus bifidus.**

Substrate or product	*Actinomyces* 10048 Anaerobic		*Actinomyces* 10049 Anaerobic		*Actinomyces* 10049 Aerobic		*L. bifidus* Anaerobic	
	μM/ml	Cts/min. /μM	μM/ml	Cts/min. /μM	μM/ml	Cts/min. /μM	μM/ml	Cts/min. /μM
Glucose utilized	22.8		31.8		31.5		49.5	
CO_2 (initial)		1995.0		1995.0		1730.0		1995.0
(final)		1696.0		1867.0		857.5		1505.0
Formic acid	8.62	119.7	11.7	98.4	15.0	61.8	2.0	1.0
Acetic "	6.52	2.4	8.7	.8	13.3	.6	69.8	.0
Lactic "	24.6	120.5	43.0	72.6	29.9	34.7	49.2	.3
–COOH		118.8		72.2		34.7		—
CH_3CHOH–		1.7		.4		.0		—
Succinic acid	8.18	1169.2	11.2	1301.2	7.04	368.0	.6	529.6
–COOH		159.5		248.4		19.1		20.4
$-CH_2-$		425.1		402.2		164.9		244.6
Beta alanine (predicted)		1009.7		1052.8		348.9		—
" " (found)		1060.0		1020.0		358.0		—

* Fermentation of glucose was done by fermentation procedure described by Pine and Howell(1) using 1% glucose casitone medium with starch. The procedure for aerobic fermentation is given in text.

with one exception. An apparently non acidic single band of high radioactivity was found running behind and not quite touching the succinate band from the aerobic #10049 fermentation of Table II. The identity of this compound has not as yet been determined. The radioactive compounds were eluted from the paper with water. The samples of succinate so obtained from the *Actinomyces* fermentation were acidified with one or 2 drops of 25% HCl, evaporated to dryness on steam bath, and sublimed *in vacuo*. Melting points were taken and all melted within 2 degrees of an authentic sample of succinic acid and with no depression observed with mixtures of the two. The succinic acid of the *L. bifidus* fermentation was not sublimed but was degraded directly. Lactic acid samples were passed through Dowex 50 resin to convert them to free acid, and were neutralized with $Ba(OH)_2$. Succinic acid was wet ashed using the reagents of Van Slyke *et al.*(11) to determine the average specific activity of its carbon atoms. A sample was then decarboxylated and the specific activity of the carbon dioxide determined according to the procedure of Phares(7) maintaining the temperature at 70°C for one half hour or for one hour at 45°C. Under our conditions, approximately 5 to 7% of the methylene carbons of known succinate-2, 3-C^{14} was obtained as the carboxyl carbon. Attempts to isolate ethylene diamine according to the procedure of Phares and Long(12) failed. Upon the suggestion of Dr. Richard Hendler, National Heart Institute, National Institutes of Health, analyses were made for amino acid in the reaction mixture. An amino acid, presumably beta alanine (13) was found. The reaction mixture was neutralized with 50% NaOH, a few ml of water added, and the Na_2SO_4 was allowed to crystallize out over night at 5°C. Ten ml of ethanol were added, the suspension was centrifuged and the supernatant was removed. The precipitate was washed twice with alcohol, and the alcohol fractions combined. The presence of beta alanine was shown using paper chromatograms run with the acetic acid-butanol-water solvent of Calvin *et al.*(14) and the ethanol-ammonia-water solvent. The former solvent was used to separate the beta alanine from residual succinic acid and trace amounts of ethylene-diamine which were also formed. The beta alanine was eluted with water from such chromatograms; its concentration determined using the ninhydrin procedure of Troll and Cannan(15); and its specific activity was determined by plating and counting directly known amounts of the amino acid. Barium lactate was decarboxy-

lated using chromic acid(16). The residual acetic acid was steam distilled, neutralized with $Ba(OH)_2$ and counted as barium acetate, applying the same correction factors used for barium carbonate.

Results. In initial experiments using *Actinomyces* strain #10049 both the volatile and non-volatile acids were radioactive. A small decrease in the specific activity of the carbon dioxide occurred during the fermentation. Later fermentations using strains #10048 and #10049 and *L. bifidus* showed that each of the 3 fixed from one to 3% of the added counts into the cells and that the volatile acids formed by *Actinomyces* were radioactive while those formed by *L. bifidus* were not. All organisms had incorporated radioactive carbon dioxide into the non-volatile acids but the total counts in this fraction from *L. bifidus* were low. An analysis was then made of a fermentation by *Actinomyces* strain #10049, the results of which are presented in Table I. Again there was observed a small decrease in the specific activity of the carbon dioxide although a net fixation of carbon dioxide occurred. The proportion of formate to acetate to succinate formed was approximately 1/1/1 but the ratio of carbon dioxide fixed to any of these products was approximately 1.54. Relatively low carbon recovery (91%) and a high redox index (1.6) indicated the formation of other products having a high redox value. Attempts to find such products failed and revealed only an additional 0.5 micromole of pyruvate formed per ml. Apparently more carbon dioxide was fixed than was required for the operation of the Wood-Werkman reaction. The specific activity of the succinate was slightly higher than that of the initial carbon dioxide. Upon degradation of the succinic acid, approximately 46% of the total activity was found in the methylene carbons. Isolation of the beta alanine verified these results.

The fermentations were repeated using *Actinomyces* strains #10048 and #10049 and *L. bifidus*. An aerobic fermentation of #10049 was also included as described above. The results are given in Table II. In the 2 anaerobic *Actinomyces* fermentations, the ratio of activity of methylene carbons to carboxyl carbons of the succinic acid was 2.67 and 1.62 for strains #10048 and #10049, respectively. For the aerobic fermentation by *Actinomyces* #10049 and the anaerobic *L. bifidus* fermentation the ratio increased to 8.6 and 12.0, respectively. No significant activity was found in any other products of *L. bifidus*.

In the anaerobic *Actinomyces* fermentations, formate and the lactate carboxyl were of approximately the same specific activity but much lower in specific activity than the final carbon dioxide. The specific activity of the final carbon dioxide in the aerobic fermentations was one half its original activity. Similarly, formate and lactate carboxyl carbon of this fermentation had a specific activity one half that found in the sister anaerobic fermentation. The lactic acid of these fermentations was labeled essentially only in the carboxyl carbon.

In aerobic *Actinomyces* fermentation it appeared that lactate was oxidized to acetate and carbon dioxide with a subsequent decrease in the amount of succinic acid formed. Little fixation of carbon dioxide into the carboxyl carbons of succinic acid occurred in this case. However, the reduction of specific activity in the methylene carbons remained proportional to the decrease in specific activity of the carbon dioxide and again was approximately one half that found in the anaerobic fermentation. In general, the calculated values of specific activity for the beta alanine were approximated by the observed values with the exception of the beta alanine isolated from the succinate of *L. bifidus*. In this case, however, the sample of beta alanine was heavily contaminated with salt and no attempt was made to purify it further.

The results of the fermentation of glucose by *L. bifidus* as shown in Table II may be expressed as being essentially: glucose→lactic acid + 1.41 acetic acid + unknown products. The results obtained with the carbon dioxide-C^{14} showed no incorporation of radioactivity into these products. That the added carbon dioxide was available to and was metabolized by this organism was shown by the high specific activity of the succinic acid which it formed. These results are in

agreement with those of Kuhn and Tiedemann(17) who found no significant incorporation of carbon dioxide into lactic and acetic acids. These workers fermented glucose-1-C^{14} with *L. bifidus* and obtained lactic and acetic acids having the same specific activity. They did not degrade these products, however. It would appear therefore that the formation of acetic acid from glucose by this organism involves a molecular rearrangement without the concomitant fixation of carbon dioxide as occurs in *Clostridium thermoaceticum*(4,5) or in *Butyribacterium rettgeri*(18).

It is of interest to note the similarity of labeling of succinic acid found in the *L. bifidus* fermentation and the aerobic fermentation by *Actinomyces* #10049. Similarities of the glucose fermentation by these organisms have been reported previously(1). It is also noted that not only do these fermentations of glucose by *Actinomyces* differ qualitatively from the fermentations of the genus *Lactobacillus* (which do not generally form succinic acid) and from members of the genera *Corynebacterium* and *Propionibacterium* (which form propionic acid) but they also differ from *Propionibacterium* in the mechanism by which they utilize carbon dioxide for succinate synthesis.

It is believed that this is the first report of the net fixation of carbon dioxide with its major incorporation into the methylene carbons of succinic acid. However, the results are similar to those reported by Jefferson *et al.* (19) who found that washed suspensions of *Rhizopus nigricans* fermented glucose in the presence of formate-C^{14} with the formation of succinic and fumaric acids having the greatest radioactivity in the central carbons. However, in this fermentation, lactic acid and ethanol formed had their greatest radioactivity in the methyl carbons while in the presence of carbon dioxide-C^{14} only carboxyl labeled acids were formed. Results similar to those of Jefferson *et al.* were obtained with *Escherichia coli* by Nutting and Carson(20).

Summary. Fermentations of glucose in the presence of carbon dioxide-C^{14} were carried out with strains of *Actinomyces* under aerobic and anaerobic conditions and with *Lactobacillus bifidus*. Fermentation products were isolated and degraded. It was found that succinic acid formed by these organisms had a high percentage of its radioactivity in the methylene carbons. When one strain of *Actinomyces* was grown in the presence of oxygen, 90% of the total activity of the succinic acid was found in the methylene carbons as compared to 62% when it was grown anaerobically.

The technical help of Mr. Carl L. Peacock is gratefully acknowledged.

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Received September 4, 1956. P.S.E.B.M., 1956, v93.

A Simple Test for Erythropoietin in Deproteinized Plasma Extract.* (22791)

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The effectiveness of a deproteinized plasma extract from rabbits made anemic with phenylhydrazine in producing erythropoietic stimulation has been questioned by several investigators(1,2). Others(3,4,5,6) have confirmed its erythropoietic stimulating properties by a variety of technics. Most recent publications on the subject have shown little rise in Hb or hematocrit in animals receiving the material but have demonstrated statistically significant rises in total red cell volume, red blood count, and nucleated red cells within the marrow. The above technics are cumbersome in that they require long-term injections of recipient animals (2-6 weeks) and require large amounts of extract. Moreover the results, though statistically significant, are not clear cut.

The following method enables a quick test of plasma from an anemic source, requires only small amounts of plasma and enables cross-species examination of the erythropoietic factor in the plasma. Also, utilizing uptake of Fe^{59} into red cells as the criterion for erythropoietic stimulation, the method requires only 3 or 4 days of subcutaneous injections of the deproteinized plasma extract and produces a clear cut differential in Fe^{59} uptake between control and experimental groups of animals. The relative increase of Fe^{59} uptake into the red cell mass of experimental groups as compared with normal controls was considerably greater in hypophysectomized rats than in normal rats.

Materials and methods. a) Young adult Sprague-Dawley rats, aged 2-3 months and ranging in weight from 250-400 g were employed in the experiments utilizing normal animals. The hypophysectomized rats† were of the same age group and had been hypophysectomized for at least 40 days to insure a stable post-hypophysectomized state. They were maintained on a diet consisting of Purina laboratory chow, and bread and milk. b) White New Zealand rabbits were given 1 cc of 1% phenylhydrazine subcutaneously daily until Hb was less than 5 g. They were then bled and the plasma prepared as described previously(5). Control plasma was obtained from normal non-treated rabbits. c) The deproteinized plasma extract was administered in two ways: 1) In unconcentrated form, the extract being diluted to the original volume of plasma. 2) In concentrated form where the extract was diluted to only ⅓ the original volume of plasma. d) The normal rats received 5 cc of deproteinized extract (concentrated or unconcentrated, anemic or normal) for 4 successive days subcutaneously. One hour after the last subcutaneous injection of plasma approximately 1 μc Fe^{59} in 1 cc saline was given intravenously and 24 hr thereafter the animal was bled from the aorta. The radioactivity in the sample was measured in a Nancy Wood well-type scintillation counter. The radioactivity in an aliquot of the original Fe^{59} solution given each animal was similarly measured. Calculation of Fe^{59} uptake into red cells was carried out as previously described(7). Precuation was taken after each experiment to run hematocrits. Any animal with low hematocrit was not included in our results since animals with low hematocrits had tended to run higher iron uptakes. The hypophysectomized rats received 2 cc of plasma extract daily (concentrated or unconcentrated, anemic or normal) for 3 successive days subcutaneously. One hour after the last injection of plasma, 1 μc of Fe^{59} was given intravenously as in the normals and 48 hours thereafter they were bled.

* The authors express their thanks to Andre Bulba, Ruth Deckert, Robert Hamilton, Edward Dywinski for their technical assistance. This investigation was supported in part by research grants from National Cancer Institute, U.S.P.H.S. and the Atomic Energy Commission.

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TABLE I. Normal Rats Given Deproteinized Plasma Extract Subcutaneously from Anemic and Normal Rabbits for 4 Consecutive Days.

	% uptake of Fe^{59} in RBC, 24 hr after last inj. of plasma				
Weekly groups*	#1	#2	#3	#4	#5
Normal plasma					
Concentrated		35.9	54.2	35.6	66.1
		28.2	56.2	41.4	58.8
		24.3	50.2	56.9	68.0
		32.9	56.3	44.8	71.3
		27.5	49.7	29.5	67.1
		36.1	50.0		47.6
Avg %		30.8	52.7	41.6	63.1
Regular	58.1		57.1	51.5	51.9
	59.8		56.3	43.5	61.4
	39.0		47.7	57.1	56.2
	61.9		49.1	61.3	54.2
	65.6	—	29.8	51.6	52.3
	59.5		53.1	46.7	55.6
	52.1				
	54.8				
	61.4				
	50.5				
	63.8				
	65.7				
Avg %	57.7		48.9	51.9	55.0
Anemic plasma					
Concentrated		47.8	80.6	69.7	67.4
		24.9	91.6	74.1	74.4
		40.0	88.7	78.1	62.2
	—	47.8	88.2	69.7	76.0
		41.8		77.2	64.1
		45.5		70.7	93.1
Avg %		41.3	87.3	73.3	72.9
Regular	69.6		84.4	55.8	64.5
	83.4		71.8	61.8	71.1
	62.5		81.0	40.2	60.8
	69.6		88.5	67.7	50.2
	67.0	—	92.6	60.4	58.0
	74.2		79.6	53.4	53.0
	69.6				
	71.1				
	51.4				
	63.4				
	47.5				
	76.0				
Avg %	67.1		83.0	56.6	59.6

* Weekly groups #1, 2, 3, 4, 5 represent separate experiments performed on five consecutive weeks. Separately prepared extract was administered on each successive week.

Calculation of Fe^{59} uptake into red cells was carried out in the same way as in the normal rats.

Results. The results are shown in Tables I and II. The normal rats showed considerable fluctuation in uptake values from week to week. However, those receiving anemic plasma extract were consistently higher than those receiving normal extract. It should be noted that the groups receiving concentrated anemic extract in all instances had higher average uptake values than the groups receiving unconcentrated anemic material. Also, the concentrated anemic extract was more consistently effective than the unconcentrated material in producing a greater relative response in experimental groups as compared with normal controls. We are unable to explain the wide week to week fluctuation in Fe^{59} uptake in these normal animals.

The hypophysectomized animals showed very distinct differences between the groups receiving normal plasma extract and those re-

TABLE II. Hypophysectomized Rats Receiving Regular and Concentrated Deproteinized Plasma Extract from Normal and Anemic (Phenylhydrazine) Rabbits Subcutaneously for 3 Consecutive Days.

	% uptake of Fe^{59} in RBC, 48 hr after last inj. of plasma		
Weekly groups*	#1	#2	#3
Normal plasma			
Concentrated	.9	1.0	1.5
	1.0	1.2	1.9
	15.4	1.3	9.3
	2.2	4.4	3.0
	7.8	5.2	6.8
Avg %	5.5	2.6	4.5
Regular	3.3	3.6	5.2
	7.8	3.6	1.1
	3.6	6.0	1.3
	1.0	1.7	1.6
		8.8	2.1
Avg %	3.9	4.7	2.3
Anemic plasma			
Concentrated	28.8	26.9	27.6
	45.9	26.8	27.2
	22.0	27.1	27.5
	49.3	21.7	82.0
	26.8	22.9	47.2
		24.6	
Avg %	34.6	25.0	42.3
Regular	18.3	22.7	25.2
	7.5	17.8	21.7
	6.6	14.5	12.6
	12.7	25.8	
	11.7	23.9	
		36.0	
Avg %	11.4	23.5	19.8

* Weekly groups #1, 2, 3 represent separate experiments performed on 3 consecutive weeks. Separately prepared extract was administered on each successive week.

ceiving anemic plasma extract. These differences were considerably more marked than those seen with the normal rats. Groups receiving concentrated anemic plasma had 6 to 9 fold increases in Fe^{59} uptake as compared with those receiving concentrated normal plasma. Likewise, groups receiving unconcentrated plasma had 3 to 8 fold increases as compared with those receiving normal plasma. Again the concentrated material was somewhat more effective than the unconcentrated in stimulating increased Fe^{59} uptake in recipient animals.

Discussion. The above data demonstrate conclusively as judged by Fe^{59} uptake into RBC, the erythropoietic stimulating property of a deproteinized plasma extract from anemic rabbits. Our findings also demonstrate the sensitivity of the hypophysectomized rat as a test preparation for demonstrating the presence of an erythropoietic factor in an extract particularly when incorporation of Fe^{59} into red cells is used as the index of red cell production.

The importance of the deproteinized character of this material is obvious. It enables the testing of erythropoietic activity of plasma from one species into another. More particularly, testing of human plasma from anemia of various sources with regard to its erythropoietic stimulating properties may be possible.

It is of interest that those investigators who have found the deproteinized material inactive have given it intravenously(1,2).† Those, on the other hand, who have given it subcutaneously have found it to be effective(3,4, 5,6).

The present results with deproteinized plasma extract from rabbits made anemic with phenylhydrazine demonstrated comparable levels of stimulation to those reported by Freid *et al.*(8) using whole plasma from anemic rats given intravenously to hypophysectomized rats. The criterion of stimulation was the same being the uptake of Fe^{59} in red cell mass.

Summary and conclusions. A simple test for erythropoietin in deproteinized plasma extract is described. A total of 3 or 4 days is required for subcutaneous injection of the plasma extract and only one intravenous injection of Fe^{59} is necessary. When concentrated plasma extracts are given to hypophysectomized rats, 6 to 9 fold differences in response between anemic and control groups are achieved. The deproteinized nature of the material enables cross-species examination of the erythropoietic factor.

† Recently, in our laboratory, hypophysectomized rats receiving intravenous injections of deproteinized rabbit anemic plasma extract (regular or concentrated) manifested a definite erythropoietic stimulation of a similar magnitude to those receiving this material subcutaneously.

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Received September 4, 1956. P.S.E.B.M., 1956, v93.

Intracellular Behavior of Brucella Variants in Chick Embryo Cells in Tissue Culture.* (22792)

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Ability of smooth brucellae to localize intracellularly is well known. Brucellae within leucocytes are protected *in vitro* against high concentrations of antibiotics and antiserum for several days(1,2). It has recently been reported, however, that monocytes derived from immune animals restrict intracellular multiplication *in vitro*(3). Non-smooth variants of *Brucella* have not been studied with regard to intracellular residence, although the selective effects of metabolite accumulation and lowered oxygen tension *in vitro* have been thoroughly investigated(4-9). Suppressive effect of selective serum (SS) factor on non-smooth brucellae *in vitro* has also been extensively studied(10). Non-smooth brucellae do not persist long *in vivo* except under conditions where selective metabolites may accumulate, such as in abscessed tissue or aborted fetal material(11-13). Thus, they have been generally considered to be of little significance in brucellosis. However, several instances of mutation from smooth to mucoid *in vivo* have recently been demonstrated, in which the mucoid variants were recovered as often from unabscessed as from abscessed tissue(14).

The present study is concerned with the selective effect of an intracellular environment upon smooth and non-smooth variants of *Brucella*.

Materials and methods. Tissue culture. The culture vessels were 16 x 125 mm screw-cap test tubes coated on the inside with a thin film of formvar (polyvinyl formal) according to the method of Barski *et al.*(15). Each culture tube contained 1.5 ml of medium consisting of 20% fresh rabbit serum, 20% chick embryo extract (1/1) and 60% Hanks' balanced salt solution. Ten or 11-day chick embryos (with head, legs and wings removed) were aseptically ground to a fine liquid pulp, and approximately 0.01 ml of this tissue was placed into each culture tube. The tubes were incubated at 37°C in an almost horizontal stationary position. Within 24 hours a heavy growth of fibroblasts covered the lower walls of the tubes. The medium was renewed every 2 or 3 days. *Bacterial cultures.* Brucellae were grown and maintained on *Brucella* agar (Albimi), enteric bacilli on MacConkey agar (Difco) and streptococci on 5% sheep blood agar. The smooth strains of *Brucella* used were *Brucella abortus* a77, a5 (CO_2 dependent) and strain 19 (Lederle no. 1952a); *Brucella suis* s101, s99 and s25, and *Brucella melitensis* m62. I (intermediate), R (rough), M (mucoid) and S^R (smooth-rough) variants of each species were obtained by prolonged growth in *Brucella* broth (Albimi) containing 300 μg/ml DL-α alanine. The acriflavin test(6) was used in conjunction with anti-S antiserum and with colonial observation(4) for differentiation of variants. The streptococcus used was a group A, β hemolytic *Streptococcus pyogenes* freshly isolated following four consecutive passages in mice. The enteric bacilli were laboratory strains of *Escherichia coli, Alcaligenes fecalis* and *Salmonella typhosa. Infection of tissue cultures.* The fibroblasts were infected on second day of culture by addition of approximately 10^6 bacteria to the tissue culture medium. Five hours later streptomycin was added to a final concentration of 10 μg/ml to kill extracellular bacteria and to prevent further extracellular growth in the medium. For the streptococcal work 1 unit/ml of penicillin was substituted for streptomycin. For the faster-growing streptococcus and enteric bacilli an infection period of only 2 hours was allowed before addition of antibiotic. At daily intervals after cell infection, a roughly quantitative determination of viable bacteria within the cells was made by scraping a platinum loopful (1/200 ml) of infected tissue from walls of tube and streaking it evenly

* Aided by grant from Research Board of University of California.

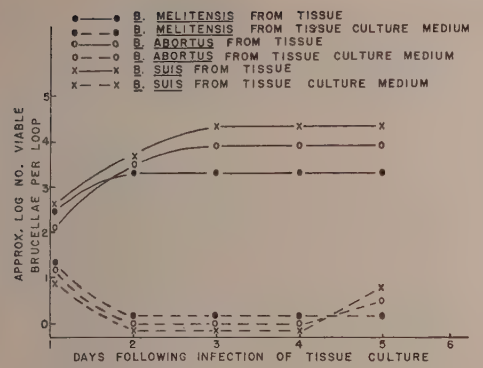

FIG. 1. Three typical experiments showing viable brucellae recovered from infected tissue as compared to those recovered from tissue culture medium in the same tube. The few bacteria appearing in the medium about the fifth day are due to degeneration of some of the infected cells.

on agar plate. A loopful of medium from the same tube was also plated out as a control against presence of extracellular bacteria.

Results. Smooth strains of all 3 species of *Brucella* entered the fibroblasts and not only survived but multiplied therein as shown in Fig. 1. If, however, the tissue cultures were heat-killed (56°C—1 min) either before or after infection, the cells were no longer capable of protecting the brucellae from the streptomycin, and no viable brucellae could be recovered after 30 hours. Thus, under the conditions of the experiment all viable bacteria recovered from tissue cultures represent growth or survival within living avian cells. When formvar-coated cover slips were included in the culture tubes, then withdrawn at intervals and stained by Perrins' modification of the Goodpasteur stain(16), intracytoplasmic bacteria were seen within a small percentage of the fibroblasts. Some fibroblasts contained only a few bacteria after several days infection, while others were nearly filled. To see whether this ability to grow within fibroblasts is unique to brucellae, several other bacterial species were studied under the same conditions. Fig. 2 shows that although all 5 species were phagocytized and thereby survived for a day or two, only the 2 intracellular species *S. typhosa* and *B. suis* multiplied or survived for long.

Prolonged culture of Brucella-infected tissue. Tissue cultures infected with brucellae did not seem to be grossly damaged by this infection. Therefore, prolonged subculture of infected fibroblasts was attempted to determine whether the host cells or the parasite would ultimately prevail over the other. In this experiment the infected fibroblasts were transferred to new tubes every 4 days following trypsinization and washing. Fibroblasts infected with *B. melitensis* m62 and *B. abortus* a5 and a77 were maintained through 7 transfers for 32 days and were still moderately infected when the subcultures were discontinued. Apparently only lightly infected and uninfected fibroblasts survived each trypsinization, since the number of viable brucellae recovered dropped following each transfer, then increased again before the next transfer. Fibroblasts infected with *B. suis* s101 became very granular after 4 to 6 days of infection, became heavily vacuolated and did not survive the second or third trypsinization. The slower growing *B. suis* strains s25 and s99 also eventually destroyed the fibroblasts they infected, although more slowly (after 3 or 4 transfers or 12 to 16 days). Over 10^4 viable brucellae per loop were recovered from cells showing cytopathogenicity due to each of the 3 B. *suis* strains. Prolonged intracellular passage of all 3 species enhanced their colonial smoothness. The colonies recovered developed

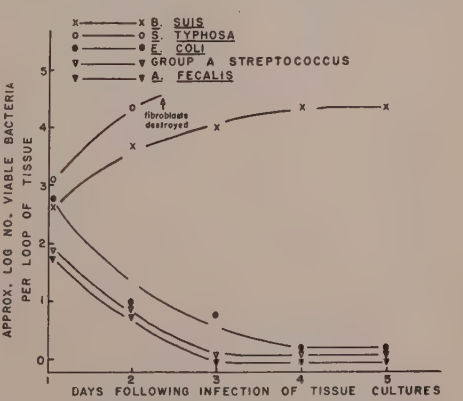

FIG. 2. Comparison of intracellular survival of several bacterial species. *S. typhosa* grew so rapidly intracellularly that cytopathogenicity occurred and streptomycin resistant mutants appeared in about 2 or 3 days.

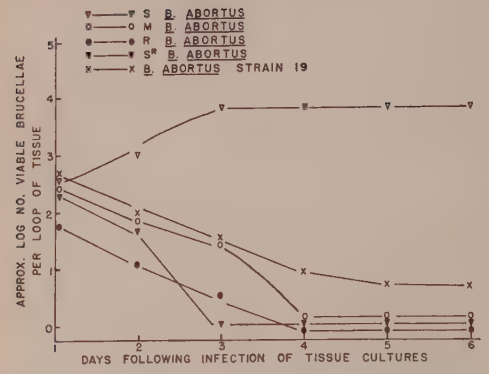

FIG. 3. Intracellular growth of smooth brucellae as compared to non-smooth variants and strain 19 in separate tissue cultures.

more slowly, were less granular and more transparent and bluish.

Intracellular behavior of non-smooth brucellae. M, R, and S^R variants from each of the 3 species were examined for their ability to grow intracellularly as did the smooth parent strains. Strain 19 was also tested. The results of a typical experiment are seen in Fig. 3. It is obvious that neither the non-smooth variants nor strain 19 multiplied or remained viable for long intracellularly. Similar results were obtained with non-smooth variants of *B. melitensis* and *B. suis*. Cells were also infected with mixed inocula of smooth and non-smooth types in various proportions. In all cases the smooth type showed a marked selective advantage (Fig. 4). In another experiment the slimy sediment from a thirty day broth culture of *B. melitensis* was used as an inoculum, and although only non-smooth variants were present on the first 3 days, smooth variants predominated on the fourth day after infection. We next attempted to obtain dissociation from non-smooth to smooth in tissue culture, followed by selection of the smooth type. Therefore, very heavy inocula of non-smooth variants were used to increase the probability of obtaining the S mutant. In some cases this was successful. S colonies were quite often obtained from I inocula and more rarely from M inocula, but never from R or S^R inocula. However, it was observed that very large inocula (over 10^7) of even non-smooth variants caused overwhelming infection, often leading to cytopathogenicity and even to prolonged survival of non-smooth types within the fibroblasts.

Effect of Brucella antiserum and of α alanine in tissue culture medium. It was felt that the presence of the SS factor(10) in the normal rabbit serum of the tissue culture medium might have influenced the results. Therefore, fresh antiserum from rabbits infected with *B. melitensis* was substituted for normal serum. Despite the presence of anti-S antibodies and complement, and despite the absence of the SS factor, the results were not altered. The S type still multiplied intracellularly and the non-S variants were eliminated. Likewise, the addition of DL-α alanine to the tissue culture medium in a final concentration of 300 μg/ml [sufficient to cause rapid selection of non-smooth variants in liquid culture(17)] did not alter the smooth selective character of the intracellular environment.

Discussion. It is apparent that brucellae and other bacteria capable of intracellular growth are in some way resistant to those intracellular mechanisms which inhibit other bacteria such as *E. coli* or the streptococcus. Possibly the loss of surface antigens(18) accompanying mutation from S to non-S might help explain the increased vulnerability of non-smooth brucellae to cellular defenses.

The survival of *Brucella* for long periods

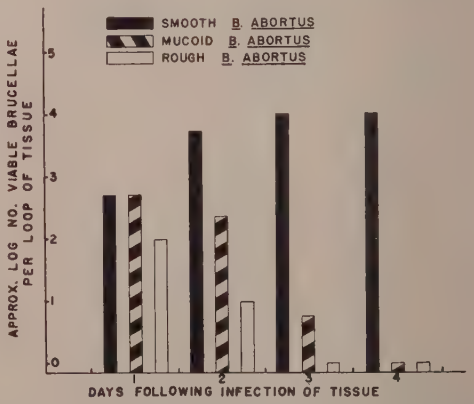

FIG. 4. Intracellular selection of the smooth type in a typical tissue culture infected with a mixed inoculum containing approximately 10^6 each of S, M, and R variants.

within fibroblasts cultured in a bactericidal medium, without gross destruction of the tissue, helps explain the capacity of *Brucella* to cause insidious, chronic disease. This is in contrast to *S. typhosa* which also multiplied intracellularly but which caused rapid cytopathogenicity.

The unaltered smooth selective capacity of fibroblasts cultured in antiserum (lacking in SS factor), indicates one reason why animals lacking the SS factor, and even immune animals, clear non-smooth brucellae from their body much more rapidly than they do the smooth type(14).

It appears that the virulence of *Brucella* is correlated with their ability to multiply intracellularly since the avirulent non-smooth variants and strain 19 are unable to do so. Further work is in progress on the intracellular infection of mammalian cells derived from normal and immune animals.

Summary. 1) Smooth brucellae were ingested by, and multiplied within, fibroblasts in tissue culture. Non-smooth variants and strain 19 neither multiplied nor survived long intracellularly unless the cells were massively infected. Brucellae remained viable intracellularly for over 30 days despite the presence of a bactericidal concentration of streptomycin in the tissue culture medium. Extensive cytopathogenicity was not evident except with *Brucella suis* which caused slow destruction of the fibroblasts. Under the same conditions of culture *Salmonella typhosa* multiplied rapidly intracellularly and caused complete cell destruction within several days. 2) Mixed infections of fibroblasts with smooth and non-smooth brucellae showed the intracellular environment to be highly selective for the smooth type. Mutation from I to S and M to S, followed by selection of the S type, occurred in several heavily inoculated tissue cultures. The addition of anti-S antiserum or DL-α alanine to the tissue culture medium did not affect the smooth-selective behavior of the intracellular environment.

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Received October 29, 1956. P.S.E.B.M., 1956, v93.

Lack of Hematopoiesis in Tail Bones Transposed to Abdominal Cavity of Hypophysectomized Rats.* (22793)

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Huggins and Blocksom(1) demonstrated that hematopoiesis could be stimulated in the normally inactive terminal tail bones of rats if the ends of the tails were transposed to the abdominal cavity. They had previously(2) shown that the subperiosteal temperature of the terminal vertebrae of the tail was approximately 7 to 12°C lower than the intra-abdominal temperature. They interpreted the establishment of hematopoiesis in the transposed tail bones to be the result of thermal stimulation. Hypophysectomy is known to have a deleterious effect upon peripheral blood and blood-forming organs. Hypophysectomized rats develop a slightly microcytic-hypochromic anemia with little alteration of the total white cell count. The bone marrow of hypophysectomized rats is hypoplastic showing an increase in fat, a decrease in the total number of nucleated cells per mm^3, a reduction in the total number of nucleated erythroid elements per mm^3, and, in the Wistar strain, little effect on white cell precursors (3). It has been shown that the marrow of the hypophysectomized rat is capable of responding to various stimuli similar to the normal rat. An erythropoietic response can be elicited in the marrow of hypophysectomized rats by anoxia, but the anoxic stimulus must be greater than that which will stimulate normal animals(4). The marrow of hypophysectomized rats is also capable of regenerating red blood cells following hemorrhage, but will form only enough to bring the peripheral count back to the pre-bled hypophysectomized levels(5-7). These examples show that even though the marrow is affected by hypophysectomy, a potential to respond to stimuli in a manner similar to normal remains insofar as erythropoiesis is concerned.

The present experiment was conducted to test whether or not hematopoiesis could be initiated in the yellow marrow of the terminal tail vertebrae of hypophysectomized rats, as it is in normal animals, when the tail is transposed to the abdominal cavity.

Material and methods. Adult female rats 3-4 months of age of the Wistar strain were used in the experiment. The animals were fed standard Purina chow supplemented once a week with lettuce. The procedure for transposition of the tails was as follows: A small incision was made in the lateral body wall; a circumferential cut was made in the skin of the tail about 3 inches from the end and the skin of the distal end of the tail was pulled off. The end of the tail was looped forward, inserted into the abdominal cavity through the lateral wall incision, and anchored in place by suturing the tendons of the tail to the muscle wall, and by anastomosing the skin of the tail with the skin of the abdominal wall. Hypophysectomies were performed by the parapharyngeal approach, and the completeness of the operation was determined at autopsy by examination of the organ site under a dissecting microscope. A small sample of blood was removed from the heart, heparinized and studied every 10 days throughout the experiment. Erythrocyte counts were done in duplicate, using U.S. certified blood pipettes and the improved Neubauer counting chamber. Hemoglobin determinations were made in a Klett-Summerson photoelectric colorimeter. Hematocrit determinations were made with Van Allen hematocrit tubes (no diluent) and spun for 1 hour at 2000 r.p.m. Rectal temperatures were taken at 10-day intervals. At autopsy vertebrae from within the abdominal cavity and from the outside loop were removed for study, the outside bone serving as a control along with bones from normal, unoperated rats. The bones were

* This investigation was supported by a research grant (A-476) from National Institute of Arthritis and Metabolic Diseases of N.I.H., P.H.S.

† With technical assistance of Mrs. Louise Brown and Mrs. Nancy Lancaster Martin.

TABLE I. Peripheral Blood Picture and Body Temperatures of Normal Unoperated Control Rats, and Normal and Hypophysectomized Rats with Tails Transposed to Body Cavity.

	Initial values				Final values			
	Erythrocyte count in millions/mm³	Hematocrit in %	Hemoglobin in g/100 cc	Rectal temp., °F	Erythrocyte count in millions/mm³	Hematocrit in %	Hemoglobin in g/100 cc	Rectal temp., °F
Normal controls (6)	8.71 ± .17*	44.7 ± .52	16.1 ± .05	100.5 ± .56	9.39 ± .28	45.0 ± .57	15.9 ± .20	100.4 ± .45
With tail transposed to abdominal cavity† (41)	8.86 ± .06	45.5 ± .33	15.9 ± .10	100.0 ± .20	8.88 ± .08	45.2 ± .35	16.4 ± .10	100.2 ± .22
Hypophysectomized; tail in cavity 70 days (16)	8.74 ± .17	46.3 ± .81	16.6 ± .26	100.4 ± .14	7.53 ± .14	34.8 ± .71	12.0 ± .22	98.6 ± .22

* ± Staud. error.
† Since no significant difference was found among any of the values for the various groups of normal animals with tails transposed, the figures for the 4 groups were combined.

fixed in Helly's fixative, decalcified for 3 days in 5% formic acid, dehydrated for 2 days in graded concentrations of tertiary butyl alcohol, cleared with xylene, embedded in paraffin, cut serially at 6 micra and stained with Giemsa stain buffered to pH 4.75.

Results. The following groups of animals were studied: (I) normal controls, (II) normal rats with tails transposed for 40, 50, 60 and 70 days, (III) hypophysectomized rats with tails transposed for 70 days, and (IV) normal and hypophysectomized rats without tail transposition kept at a high environmental temperature.

Group I—normal controls—The peripheral blood values and body temperatures for these animals are tabulated in Table I. Section of the tail vertebrae revealed a typical yellow or fatty marrow as shown in Fig. 1. There is a delicate reticulum with occasional isolated hematopoietic cells identifiable in the interstices. The vessels in the marrow may or may not be filled with blood.

Group II—normal rats with transposed tail —The rats of this group can be considered as a unit because the differences in time did not manifest any significant differences in the results. Transposition of the tail had no significant effect on the peripheral blood picture even though red marrow did develop in some of the tail vertebrae (Table I). The body temperature also showed no significant change from the beginning to the end of the experiment (Table I). Vertebrae taken from the outside loop of the tail showed the typical fatty marrow seen in the normal rat (Fig. 1 and 2). The response of the vertebrae from within the abdominal cavity is tabulated in Table II. Thirty-seven of a total of 41 operated animals (90.2%) showed a positive response, a positive response being recorded when there was any accumulation of hematopoietic cells into groups within the interstices of the reticulum. This was sometimes accompanied by an obvious thickening of the reticular framework. A response that was considered "good" is illustrated in Fig. 3. It is characterized by a substantial increase in hematopoietic cells of all types. Responses equal to this were seen at all time intervals

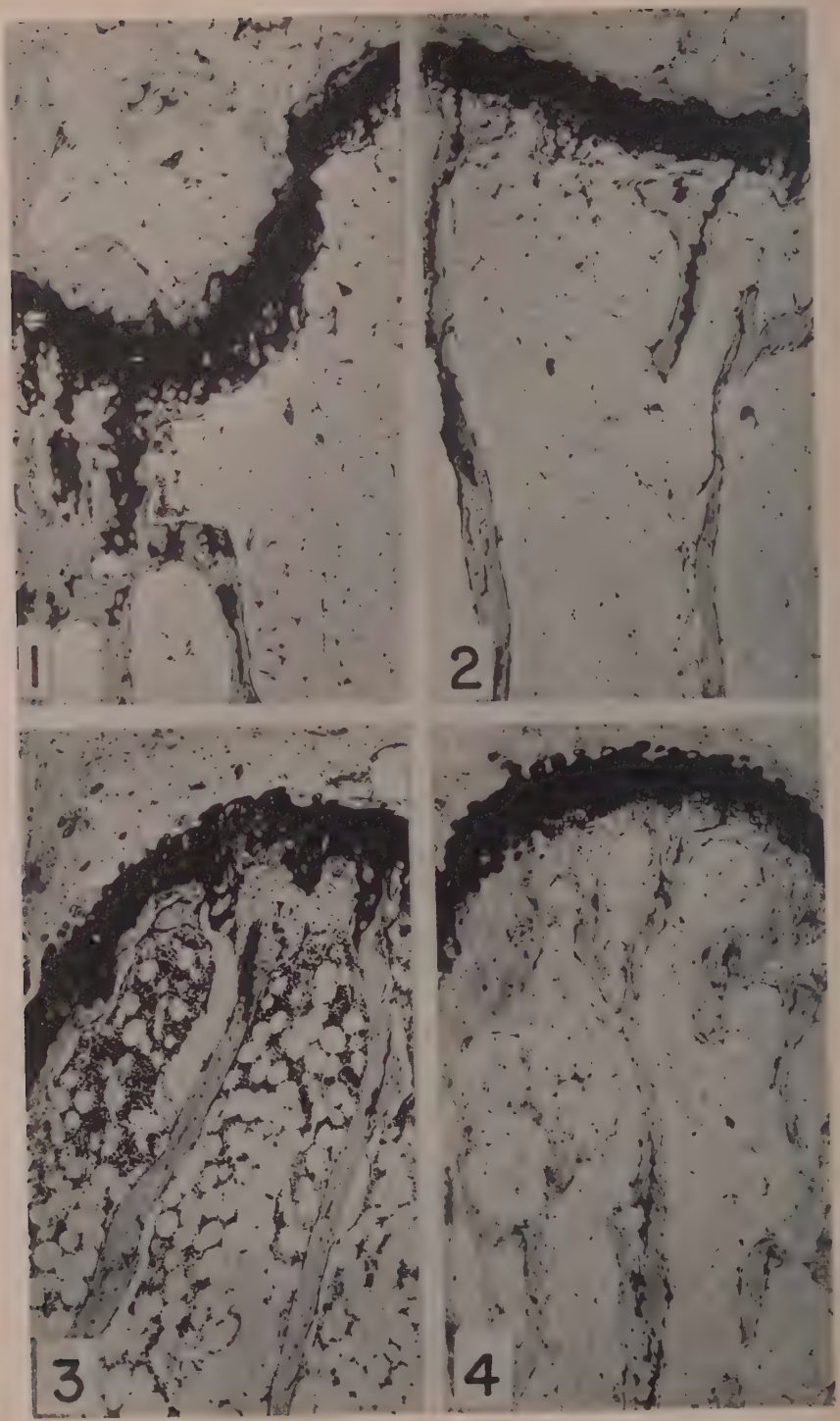
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TABLE II. Hematopoietic Response in Tail Bones Transposed to Abdominal Cavity in Normal and Hypophysectomized Rats.

	No. of rats	No. with Positive response	Erythroid elements	Myeloid elements	Megakaryocytes
Tail in abdominal cavity 40 days	10	10	10	10	9
Idem 50 "	11	9	9	9	5
" 60 "	8	7	7	7	4
" 70 "	12	11	10	11	10
Hypophysectomized; tail in cavity 70 days	16	1	?	1	0

of this operated group. In every case in which there was a response the distribution of the cells was limited to the metaphysis of the bone; increases in vascularity were noted in some but not in all of the vertebrae. Table II shows that the various cell types are well represented in the different groups of operated normal animals. The response was not limited to any one cell type; erythropoiesis, myelopoiesis, as well as megakaryocytic development, was initiated. The observations also agreed with the finding of Huggins and Blocksom in that there was a tendency for a more extensive myeloid distribution than the other cell types.

Group III—hypophysectomized; tail transposed animals—Seventy days after hypophysectomy these animals showed the usual post-hypophysectomy anemia (Table I) and a significant drop (1.8°F) in body temperature. A study of the tail vertebrae from within the body cavity of these animals revealed that only 1 (6.2%) of 16 animals showed any indication of response; the response in this 1 animal was no greater than the minimal response recorded for the control group. No megakaryocytes were seen and identification of erythroid elements was questionable. The remaining animals showed marrows similar to the unoperated control group (Fig. 4).

Group IV—normal and hypophysectomized animals at elevated environmental temperatures—Huggins and Blocksom found that hematopoiesis could be stimulated in the tail bones of normal rats kept at high environmental temperatures. A small experiment was conducted to repeat this work and to extend it to the hypophysectomized animal. Five normal and 5 hypophysectomized rats were kept in an oven at 98°F for a period of 50 days. Only 1 of the 10 rats (a normal animal) showed a response.

Discussion. Huggins and Blocksom postulated that conversion of yellow marrow to red marrow could be accomplished by thermal stimulation, and that red marrow was distributed in those bones having higher environmental temperatures and yellow in those having lower temperatures. The present experiment shows that this effect cannot be shown in the hypophysectomized animal.

Although many changes occur in the hypophysectomized animal which may account for this lack of response, lowered body temperature must be considered as a possible cause. Huggins and Blocksom found that when normal, unoperated animals were placed in an oven for 21 to 70 days, at an environmental temperature of 91 to 96°F, hematopoiesis was stimulated in the tail bones of all of the animals, but the response was not as extensive as it was in the transposed vertebrae. These temperatures are well below the body temperature of the hypophysectomized rat. This suggests, since hematopoiesis can be stimulated at these lower temperatures in the normal animal, that the lowered body temperature in the hypophysectomized rat is not the

FIG. 1. Section from metaphysis of a tail vertebra from a normal rat. ×160.

FIG. 2. Similar section of a tail vertebra taken from the loop of tail lying outside of the body after the end had been transposed to within the abdominal cavity of a normal rat. ×160.

FIG. 3. Similar section of a tail vertebra taken from that portion of tail lying within abdominal cavity of a normal animal for 70 days. ×160.

FIG. 4. Same as Fig. 3 but from a hypophysectomized rat. ×160.

factor limiting stimulation. This same observation, however, was made on only 1 of 5 animals, kept at 98°F, in the present experiment. In addition, it is known that marrow in the hypophysectomized rat is less sensitive to various stimuli, *i.e.*, anoxia(4), and bleeding(5-7); therefore, until such time as hypophysectomized rats are maintained at temperatures equal to normal or even higher than normal, the lowered body temperature cannot be eliminated as possibly having had an influence on the results.

Huggins and Blocksom also showed that anemia induced by repeated bleeding or splenectomy of animals infected with Bartonella did not stimulate hematopoiesis in the tail bones of normal rats, but the accompanying anemia resulted in a more extensive hematopoietic response in bones transposed to the abdominal cavity. The anemia of hypophysectomy does not have this stimulatory effect.

It is of interest to note that in the present experiment hypophysectomy resulted in an inhibition of both erythropoiesis and myelopoiesis. This is in contrast to the results obtained by Meineke and Crafts(3) where, in femur marrow, hypophysectomy induced a depression in erythropoiesis only; there was no effect on myelopoiesis.

It is possible that hypophysectomy has removed some hormone or caused some change in the animal which is necessary for the thermal stimulus to be effective. This could be the direct effect of removal of a single hormone (such as growth hormone), a combination of hormones, or might be the result of a secondary effect on metabolism. Reparative hormonal therapy under conditions outlined in this experiment might be of value in providing information as to the mechanism involved in the initiation of hematopoiesis.

Summary. Thirty-seven of 41 normal female rats, in which the ends of the tails were transposed into the abdominal cavity, showed a stimulation of hematopoiesis in the intra-abdominal vertebrae. Developing cells of all 3 types—erythroid, myeloid and megakaryocytes—were observed in these animals. In similarly treated hypophysectomized female rats only 1 of 16 animals showed any indication of response. The experiment provides another example of the depressed hematopoietic potential of the hypophysectomized rat.

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Received September 7, 1956. P.S.E.B.M., 1956, v93.

Resistance to Bacteria in Hemorrhagic Shock. VII. Demonstration of Leucotoxin in Plasma of Shocked Rabbit.* (22794)

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In the first of this series of studies it was shown that the phagocytic index of granulocytes from the peritoneal cavity of the normal dog, immersed in plasma from the dog in hemorrhagic shock, falls more and more the longer the dog has been in shock when the

* Aided by grant from National Heart Institute, N.I.H., Bethesda, Md. (Grant No. H-2014R)

test plasma is taken(1). This was attributed to a progressive decline in the potency of phagocytosis-promoting factors in the plasma. But it would have been just as valid to interpret the data as signifying a progressive rise in the potency of a phagocytosis-inhibiting factor in the plasma. In the present communication we report evidence that the plasma of the shocked rabbit contains a substance that inflicts functional and morphologic damage upon normal phagocytes *in vitro*.

Method. The unchallenged peritoneal cavity of a normal or shocked rabbit, when washed with iced gelatin Locke's solution, yields a cell suspension containing 70% macrophages. Thus one can obtain and compare these cells from the normal and shocked rabbit. But a comparison of granulocytes is not possible, because they appear in large numbers and as the predominant cell after several hours in response to an irritant injected into the peritoneal cavity of the normal rabbit, but they do not appear in significant numbers in response to the same stimulus in the shocked rabbit, either during shock or for several hours after an effective transfusion. The reason for the failure of granulocytes to leave the circulation in the shocked rabbit may be because a severe granulocytopenia develops soon after shock is induced and persists for some hours, even after an effective transfusion. For the same reason the blood is an unpromising source of a sufficient supply of these cells. We have, therefore, obtained data on the effect of plasma from normal and shocked rabbits upon the morphology and function of (a), macrophages from the peritoneal cavity of the normal and shocked rabbit and of (b), granulocytes from the peritoneal cavity of the normal, but not of the shocked rabbit. By a technic described elsewhere(2) macrophages were obtained from the peritoneal cavities of normal and shocked rabbits, and granulocytes were obtained from the peritoneal cavities of normal rabbits. They were promptly harvested by centrifugation for 20 minutes at 4°C, washed 3 times in iced gelatin Locke's (g-L) solution, and resuspended in a final concentration of 10 million cells per 0.1 ml of the g-L solution. Plasma was obtained from normal rabbits and from rabbits which were either in advanced shock (irreversible to transfusion), or in shock of 2 hours duration (reversible shock). In the latter it was taken at the end of the 2 hours, just before transfusion, or 4 hours after the transfusion. The g-L solution and the plasma were heparinized (4 mg/100 ml). *For study of comparative morphology of cells in normal and shock plasma,* 0.5 ml of plasma was added to 0.1 ml of cell suspension and the mixture incubated for one hour at 37°C. Smears of the mixture were then made and stained with methylene blue (for nuclei) or Wright's stain (for cytoplasm). All preparations were made in duplicate. Granulocytes were classified as mildly injured when the nucleus showed a change from the multilobular to an oval or crescent shape and a shift to the periphery of the cytoplasm. More severe injury consisted of distortion of the cytoplasm, with blurring of detail and loss of granularity, and conversion of the nucleus to a crescent shaped amorphous mass displaced to the periphery of the cytoplasm. Such cells resemble L. E. cells. The percent of injured cells was determined from counts of 200 cells in each preparation. The mildly injured cells were counted separately from the severely injured ones. Less than 2% of granulocytes freshly garnered from the peritoneal cavity and suspended in the g-L solution show mild or severe injury. After they are allowed to incubate in the g-L solution or in normal plasma for one hour at 37°C, not more than 5% show such injury. *To determine reversibility of morphologic injury* to granulocytes, the shock plasma and cell mixture was incubated at 37°C for one hour, then washed 3 x in iced g-L solution, and then resuspended in the g-L solution or in normal plasma, and again incubated for one hour at 37°C. Smears were made after the first and second incubation, and any change in the percentage of injured cells noted. *For study of comparative phagocytic and bacteriostatic power of phagocytes* in normal and shocked plasma the following technic, adapted from Wood and Smith(3), was employed: 0.1 ml of cell suspension was added to 0.01 ml of a 5 hour culture of Friedlander's bacillus (about one million bacteria). To this mixture 0.1 ml of normal or shock

plasma was added. (Since storage for some days at –20°C was found not to change the effect of plasma on the behaviour of phagocytes, the test plasma was either fresh or stored.) Aliquots (0.015 ml) of this mixture were incubated on 1 cm squares of filter paper in a moist Petri dish at 37°C for one hour. One square was then smeared on a glass slide, and the latter stained with methylene blue. The percentage of bacteria ingested, and the percentage of cells with ingested bacteria were determined by counts of the intracellular and extracellular bacteria in fields containing a total of 200 cells. The remaining filter paper preparations were pooled and washed until clean with 0.5 to 1.0 ml of iced g-L solution. The washings were transferred to a centrifuge tube and spun for 5 minutes at 800 rpm in a refrigerated centrifuge to precipitate the cells. The supernatant was decanted and spun for 15 minutes at 2500 rpm to harvest the uningested bacteria. The cell precipitate was triply washed in iced g-L solution to remove uningested adherent bacteria. The washed cell precipitate, to which 1 ml of iced g-L solution was added, was then transferred to a mortar containing sterile sand, and ground for 15 seconds in order to rupture the cells and release the ingested bacteria. The mixture was transferred with added iced g-L solution to a centrifuge tube, and spun for 5 minutes at 800 rpm to remove the sand and cellular debris. The ingested bacteria were now free in the supernatant, which was decanted and spun at 4°C for 15 minutes at 2500 rpm to harvest these bacteria. Separate "agar smears" were made from the precipitates of uningested and of ingested bacteria. These smears were prepared by spreading a minute sample of each precipitate on a coverslip previously coated with a layer of melted agar, then covering the smear with another layer of melted agar. The coverslips were then transferred to a covered Petri dish and incubated for 2 hours at 37°C. They were then stained with methylene blue, inverted, mounted on a glass slide and sealed with paraffin. Counts were made of the number of single bacteria (dead†) and of colonies (live) and the percent of dead bacteria determined.

TABLE I. % of Granulocytes Showing Morphologic Injury after Incubation for One Hour at 37°C in Shock Plasma* in 6 Experiments.

Reversible shock plasma, no transfusion			Reversible shock plasma, 4-6 hr after transfusion			Irreversible shock plasma, no transfusion		
M†	S‡	T§	M	S	T	M	S	T
20	50	70	15	15	30	27	57	84
16	14	30	40	15	55	20	14	34
18	15	33	20	10	30	18	14	32
23	34	57	30	30	60	18	18	36
			30	25	55	31	37	68
			20	60	80			

* Effect of normal plasma was determined in every case simultaneously with effect of shock plasma on the same cell suspension. Because normal plasma did not produce damage to more than 5% of the cells in any case, data for normal plasma are omitted.

† M—Mild injury. ‡ S—Severe injury ("L.E." cells). § T = M + S.

Some 6 determinations of the phagocytic and of the bacteriostatic activity were made in duplicate in each of the following categories: 1, normal macrophages in normal plasma; 2, normal macrophages in shock plasma; 3, macrophages from shocked rabbits in normal plasma; 4, macrophages from shocked rabbits in shock plasma; 5, granulocytes from normal rabbits in normal plasma; and 6, granulocytes from normal rabbits in shock plasma.

Results. A. *Effect of shock plasma on morphology of phagocytes.* (Fig. 1 and 2). 1. *Macrophages.* These cells appear normal when freshly harvested from the unchallenged peritoneal cavity of the shocked as well as of the normal rabbit. No change from normal was observed after standing for one hour at 37°C in either g-L solution or in normal plasma. But after standing at 37°C for one hour in plasma from rabbits in advanced shock some 20% showed damage consisting of any or all of the following changes: Small and large vacuoles in the cytoplasm, loss of definition of the cell wall, pyknosis of the nucleus, or blurring of its membrane. 2. *Granulocytes* (Table I). Not more than 1-2% of these cells show abnormal morphology when examined immediately after harvesting from

† Single bacteria might be inhibited rather than dead. But the general significance of the observation is valid, whichever is the case.

the peritoneal cavity. After these cells are immersed in g-L solution or normal plasma for one hour at 37°C, the percentage of damaged cells does not exceed 5%. Table I lists the data on plasma from reversibly shocked and irreversibly shocked rabbits. Even if one

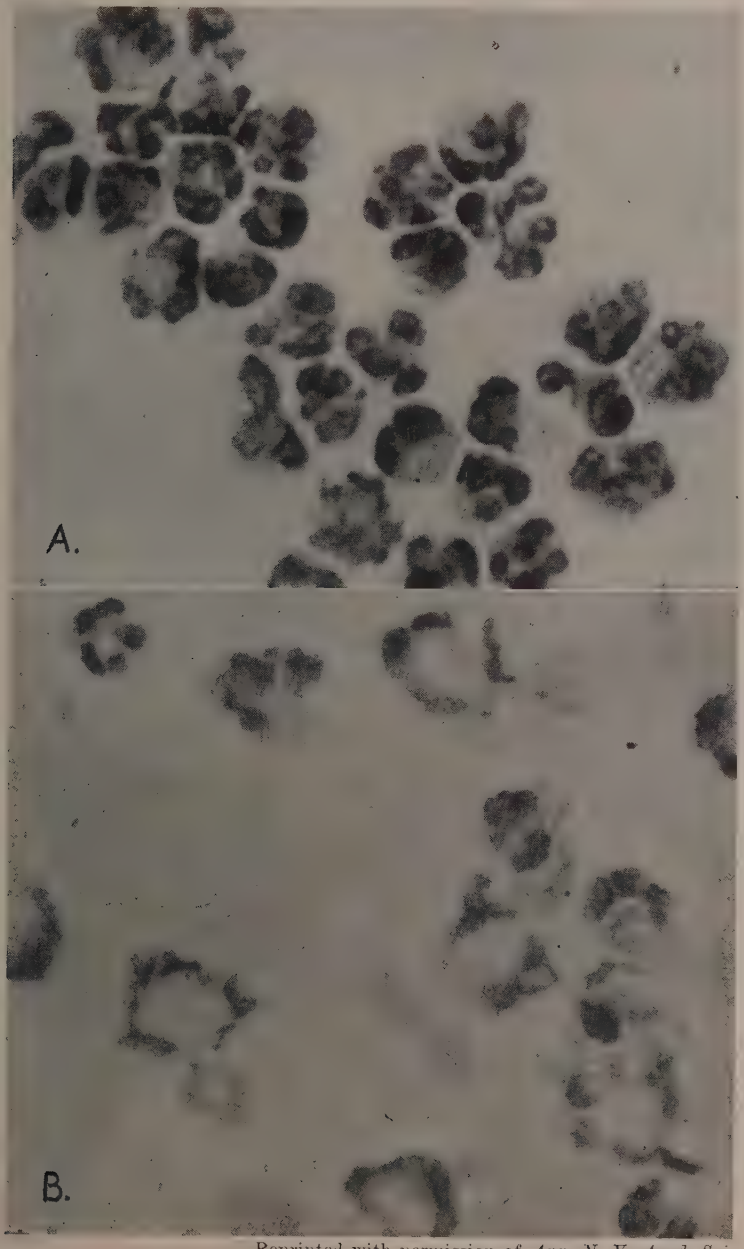

FIG. 1. A. Granulocytes from peritoneal cavity of normal rabbit washed in iced gelatin–Locke's solution, and then suspended in fresh plasma from a normal rabbit. B. Same as A, except that plasma is from a rabbit in hemorrhagic shock of 2 hr duration.

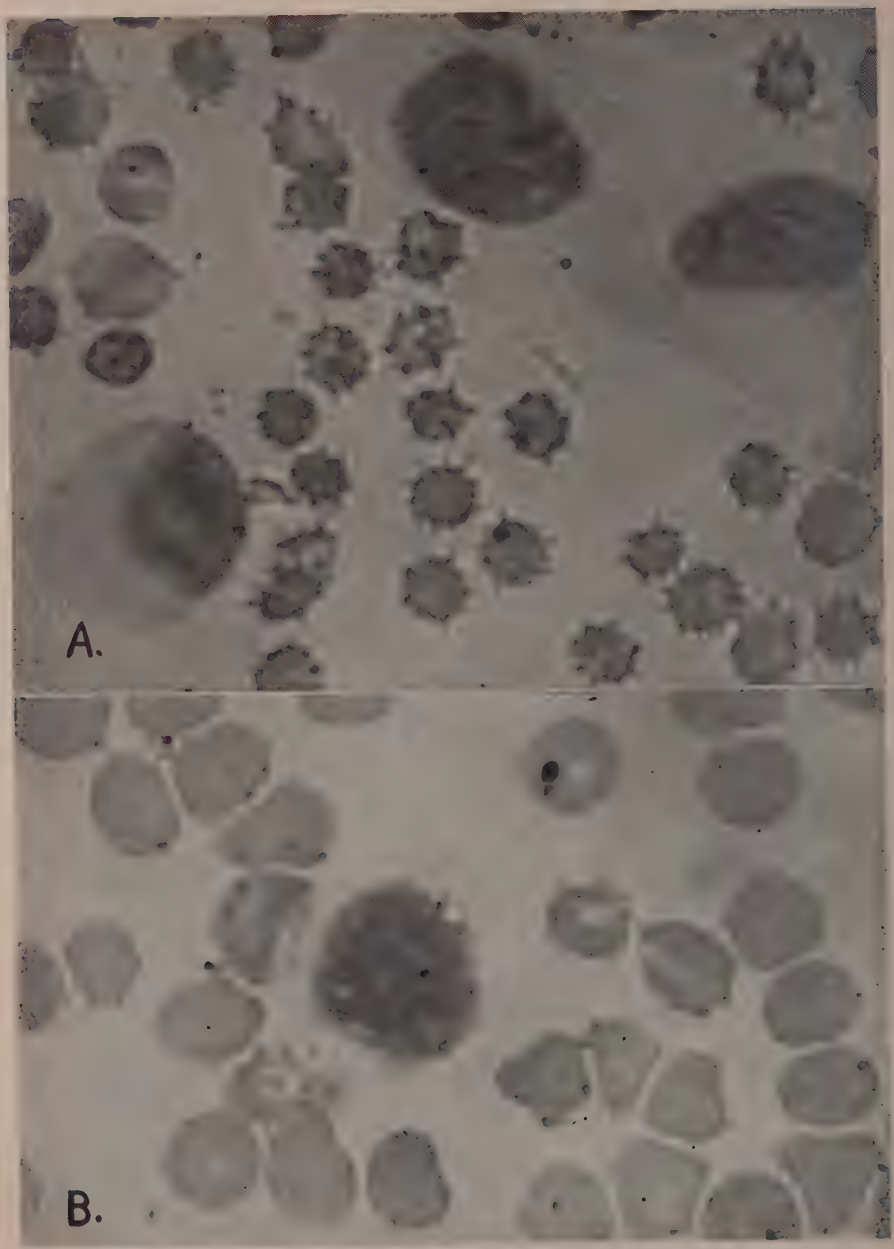

FIG. 2. A. Macrophages from peritoneal cavity of normal rabbit—washed in iced gelatin—Locke's solution and then suspended in fresh plasma from normal rabbit. B. Same as A except that plasma is from a rabbit in hemorrhagic shock of 2 hr duration.

TABLE II. % of Normal Granulocytes Showing Injury after Incubation for One Hour at 37°C in Reversible Shock Plasma followed by Incubation for One Hour in g-L Solution or Normal Plasma. 6 experiments.

Reversible shock plasma, 4-6 hr after transfusion			Gelatin, Locke's sol.			Normal plasma		
M*	S†	T‡	M	S	T	M	S	T
15	15	30	5	3	8	5	0	5
40	15	55	6	5	11	5	3	8
20	10	30	2	2	4	3	3	6
30	30	60	5	13	18	11	2	13
30	25	55	5	5	10	8	4	10
20	60	80	8	18	26	5	15	20

* M—Mild injury.
† S—Severe injury ("L.E." cells).
‡ T—M + S.

is inclined to accept the "L.E." cells only as sufficiently significant evidence of morphologic injury, there is no doubt that shock plasma is distinguishable from normal plasma by the presence in shock plasma of a cell damaging factor.

Reversibility of morphologic injury. That the morphologic damage is reversible is shown in Table II, which compares the percentage of cells damaged by irreversible shock plasma with the percentage of the same cells washed free of this plasma and subsequently exposed for one hour at 37°C to g-L solution or to normal plasma. The technic of the shock experiment, as we perform it(4), allows continuous exchange between the blood in the circulation of the animal and the blood lost by the animal into an elevated reservoir. It is conceivable that the blood, which stands in the reservoir at room temperature for several hours, may generate a toxic factor that is factitious, and that such a toxic factor, rather than one developing in the animal, accounts for the effect of shock plasma upon the integrity of the phagocytes. That this is not so is indicated by the data in Tables III and IV, which show (1), that the granulocytopenia which develops in shock does not affect the differential count of the blood in the reservoir (Table III); (2), that the number of granulocytes in the latter remains near normal (Table III); (3), that the percent of normal granulocytes damaged by the animal's plasma after 2 hours of shock is substantially

TABLE III. Comparison of Differential Leucocyte Count in Circulating Blood after 2 Hr of Hemorrhagic Shock with Differential Leucocyte Count in Blood in Elevated Reservoir.

Exp. No.	Type of cell	In circulating blood before shock	In circulating blood after 2 hr of shock	In elevated reservoir
1	P*	67	41	73
	L†	27	55	23
	M‡	6	4	4
2	P	67	31	68
	L	30	69	29
	M	3	0	3
3	P	61	19	45
	L	38	79	54
	M	1	2	1
4	P	51	34	60
	L	47	66	39
	M	2	0	1

* P—Polymorphonuclear leucocytes.
† L—Lymphocytes.
‡ M—Mononuclear cells.

greater than the percent damaged by the plasma in the reservoir (Table IVA); and (4) that the phagocytic and bacteriostatic potency of normal granulocytes immersed in plasma from the reservoir after 2 hours of shock is distinctly greater than that of the

TABLE IV-A. Comparison of % of Mildly and Severely Damaged Granulocytes Immersed in Plasma from Circulating Blood and in Plasma from Blood in Elevated Reservoir after 2 Hr of Hemorrhagic Shock. 4 experiments.

Plasma from circulating blood			Plasma from blood in reservoir		
M*	S†	T‡	M	S	T
18	15	33	20	4	24
23	34	57	6	7	13
20	50	70	3	4	7
16	14	30	10	3	13

* M—Mild injury.
† S—Severe injury.
‡ T—M + S.

TABLE IV-B. Comparison of Phagocytic and Bacteriostatic Potency of Normal Granulocytes Immersed in Plasma from Blood in Reservoir after 2 Hr and in Plasma from Circulating Blood after 2 Hr in Shock. 4 experiments.

Phagocytic index		Bacteriostatic index	
SP*	RP†	SP	RP
10	45	30	49
18	27	15	44
14	50	19	50
23	40	32	76

* SP—Reversible shock plasma.
† RP—Plasma from blood in reservoir.

TABLE V. Effect of Irreversible Shock Plasma on Percentage of Bacteria Ingested by Phagocytes.*

Normal macrophages† in			Shock macrophages‡ in			Normal granulocytes§ in		
		S.P.**			S.P.			S.P.
N.P.‖	S.P.¶	N.P.	N.P.	S.P.	N.P.	N.P.	S.P.	N.P.
35	18	.51	34	20	.60	53	26	.49
44	31	.70	37	30	.80	42	21	.50
33	20	.60	37	16	.43	50	32	.64
31	16	.51	30	17	.56	31	19	.61
55	34	.61	50	21	.42	60	27	.45
—	—	.59	—	—	.56	—	—	.54

* Phagocytic activity = % of bacteria in field of 200 intracellular phagocytes. Figures are in %.

† Macrophages harvested from unchallenged peritoneal cavity of normal rabbit immediately after killing.

‡ Macrophages harvested from unchallenged peritoneal cavity of rabbit killed 4 hr after transfusion for shock of 2 hr duration.

§ Granulocytes from peritoneal cavity of normal rabbit killed 6 hr after inj. of beef infusion broth.

‖ N.P.—Normal plasma. ¶ S.P.—Plasma from rabbit shortly before death from hemorrhagic shock, not responsive to transfusion.

** $\frac{\text{S.P.}}{\text{N.P.}} = \frac{\text{Phagocytic index in shock plasma}}{\text{Phagocytic index in normal plasma}}$.

same cells immersed in the plasma from the circulating blood taken at the same time.

B. *Effect of shock plasma on phagocytosis. Macrophages.* From Table V one can conclude that: (1) there is no significant difference between the percent of bacteria ingested by macrophages taken from the normal rabbit and the percent of bacteria ingested by macrophages from the rabbit dying of irreversible shock. (2) The percent of bacteria ingested by macrophages from the normal and shocked rabbit and by normal granulocytes is depressed to about the same extent by shock plasma, *i.e.* by somewhat less than 50%. (3) The deficient phagocytosis is due to a toxic factor in the plasma of the shocked animal. Table VI shows that the shock plasma reduces the percentage of cells with ingested bacteria. We have no data to show whether the reduced phagocytic index is due to the total suppression of phagocytosis by some cells or to a reduction in the average number of bacteria ingested per cell. To determine whether the amount of inhibiting factor depends upon the duration of shock, we have compared the degree of depression of the phagocytic indexes in the various types of shock plasma in Table VII. No significant difference between the toxicity of the plasma of reversible and irreversible shock appears in the data except those on the activity of granulocytes, which, though suggestive, are too meager to permit a definitive inference.

TABLE VII. Comparative Depression of Phagocytic Activity of Phagocytes by Plasma from Rabbits in Reversible and Irreversible Shock.*

	Normal macrophages in		Shock macrophages in		Normal granulocytes in	
	R'SP†	ISP‡	R'SP	ISP	RSP§	ISP
	.51	.56	.60	.50	.66	.49
	.70	.70	.80	.63	.74	.50
	.60	.41	.43	.46	.70	.64
	.51	.71	.56	.60	.62	.61
	.61	.59	.42	.65	—	.45
Avg	.60	.60	.56	.57	.68	.54

* Figures in each case represent ratio of phagocytic index of cells in shock plasma to phagocytic index in normal plasma.

† R'SP—Plasma taken 4 hr after transfusion for 2 hr of shock.

‡ ISP—Plasma taken from rabbits before death from advanced shock. No transfusion.

§ RSP—Plasma taken after 2 hr of shock. No transfusion.

C. *Effect of shock plasma on bacteriostatic potency of phagocytes.* Table VIII lists the percentage of ingested bacteria rendered bacteriostatic by phagocytes after immersion for one hour at 37°C in normal plasma, and in plasma from reversibly and irreversibly shocked rabbits. The depression of the bacteriostatic potency of these cells by the various shock plasmas is expressed in terms of the

TABLE VI. Effect of Shock Plasma on Percentage of Cells Containing Bacteria.

Normal macrophages in			Normal macrophages in			Shock macrophages in			Shock macrophages in			Normal granulocytes in			Normal granulocytes in		
		R'SP			ISP			R'SP			ISP		RSP				ISP
NP*	R'SP‡	NP	NP	ISP§	NP	NP	R'SP	NP	NP	ISP	NP	NP	RSP†	NP	NP	ISP	NP
40	38	.95	22	16	.73	52	20	.40	20	16	.80	30	18	.60	41	20	.49
30	19	.63	23	16	.69	28	20	.71	23	14	.61	40	30	.75	30	18	.60
33	25	.75	41	33	.80	34	18	.71	40	18	.45	33	31	.94	39	31	.80
25	25	1.00	30	27	.90	20	18	.53	28	25	.89	24	18	.75	20	18	.90
33	20	.60	24	18	.75	18	20	.90	27	18	.66	—	—	—	41	29	.70
34	25	.74	—	—	—	30	25	1.11	—	—	—	—	—	—	—	—	—
Avg		.78			.77			.73			.68			.76			.70

* NP—Normal plasma. † RSP—Plasma taken after 2 hr of shock. No transfusion. ‡ R'SP—Plasma taken 4 hr after transfusion for 2 hr of shock. § ISP—Plasma taken from rabbits before death from advanced shock. No transfusion.

TABLE VIII. Effect of Shock Plasma on Bacteriostatic Potency of Phagocytes.

Normal macrophages in						Shock macrophages in						Normal granulocytes in								
		R'SP			ISP			R'SP			ISP		RSP			R'SP			ISP	
NP*	R'SP†	NP	NP	ISP‡	NP	NP	R'SP	NP	NP	ISP	NP	NP	RSP§	NP	NP	R'SP	NP	NP	ISP	NP
49	29	.59	37	20	.54	50	30	.66	40	18	.45	68	30	.44	60	28	.47	55	27	.49
40	30	.75	—	—	—	46	25	.61	41	25	.61	51	15	.30	67	31	.46	71	30	.42
51	25	.49	50	27	.54	50	22	.44	37	20	.54	46	19	.41	57	29	.57	64	24	.37
35	25	.71	40	29	.73	30	25	.83	22	15	.68	80	32	.40	48	20	.42	51	18	.35
38	29	1.76	25	15	.60	40	32	.80	40	20	.50	—	—	—	—	—	—	68	31	.46
24	20	.59	38	21	.55	28	20	.63	—	—	—	—	—	—	—	—	—	—	—	—
Avg		.65			.59			.65			.56			.39			.47			.51

* NP—Normal plasma. † R'SP—Plasma taken 4 hr after transfusion for 2 hr of shock. ‡ ISP—Plasma taken from rabbits before death from advanced shock. No transfusion. § RSP—Plasma taken after 2 hr of shock. No transfusion.

ratio of the percentage of ingested bacteria that did not multiply when exposed to shock plasma to the percentage of ingested bacteria that did not multiply when exposed to normal plasma. These data demonstrate a considerable degree of damage to the bacteriostatic potency of phagocytes by shock plasma. They also demonstrate that the injury is about the same whether the shock is reversible or irreversible, and that the toxin in the plasma is still as active 4 hours after transfusion for 2 hours of shock as it is immediately after 2 hours of shock. Furthermore, it appears that the leucotoxin reduces the bacteriostatic potency of granulocytes to a greater degree than of macrophages.

Discussion. From the foregoing data we conclude that a leucotoxin is present in the plasma of hemorrhagic shock. This conclusion rests chiefly upon the evidence relating to the functional behaviour of the phagocytes. Since we have no data on the functional capacity of the morphologically damaged phagocytes, we cannot say whether the morphologic injury bears any relation to the functional injury. Nor can we say that the leucotoxin responsible for the functional injury is the substance causing the morphologic injury.

What is the source of the leucotoxin? The evidence given is sufficient to exclude a substance that might be produced in consequence of the special conditions of our shock experiment. The phagocytes and the plasma in the relatively stagnant blood in the reservoir have been found to be comparable in behaviour to the phagocytes and the plasma from the blood of the normal rabbit. And the transfusion has been excluded as a factor in the toxicity of the plasma of the animal recovering from shock, and of the animal dying of shock after a transfusion.

Is the leucotoxin an endotoxin? Endotoxins given intravenously are known to produce granulocytopenia. We have data to be published which show that endotoxins, added to a mixture of normal plasma and normal granulocytes in concentrations equivalent to those in circulating blood after injecting a sublethal dose, produce morphologic damage which is virtually the same as that produced by plasma from the shocked animal. Endotoxins also damage the antibacterial defense mechanisms and produce typical irreversible peripheral vascular collapse(5). Elsewhere(6) we have put forward the view that hemorrhagic shock weakens the antibacterial defense, in consequence of which unfettered bacterial activity eventually inflicts irreversible damage to the peripheral vessels and prevents them from responding to transfusion. Weakness of the antibacterial defense can presumably result from tissue hypoxia alone. But the presence of a leucotoxin in the blood must add to the damage to the antibacterial defense. Thus the way is open for the production of endotoxins from invading bacteria, whether the leucotoxin itself is or is not an endotoxin. Consequently there is reason to assume that a vicious circle is set up, and that toxin begets more toxin, with resulting injury to the peripheral vessels which is indistinguishable from that known to be caused by the direct injection of endotoxins into a healthy animal(5).

According to this thesis the toxicity of the plasma of the shocked rabbit just before death should be greater than that of the plasma of the rabbit which is still reversible to transfusion. We have already referred to evidence that this is so in the dog, but we do not find it so in the rabbit. This may be because our assay technic is too crude, or because there is in fact no necessary relationship between the amount of circulating toxin and the development of irreversibility to transfusion in the rabbit. For in this species we have found that the effect of a given amount of endotoxin on the peripheral vascular system is related not only to the dose of endotoxin, but to the sensitivity to the endotoxin, which increases enormously as the shock continues (7).

Summary and conclusions. 1) Normal macrophages from the peritoneal cavity of the normal and shocked rabbit, and granulocytes from the irritated peritoneal cavity of the normal rabbit show no significant morphologic injury after immersion in normal rabbit plasma for one hour at 37°C. But after immersion in plasma from rabbits in hemorrhagic shock, a considerable percent of the

cells show morphologic injury. A large number of granulocytes show changes similar to those characteristic of the L.E. cells. These changes are reversible if the shock plasma is replaced by normal plasma. 2) Shock plasma produces a severe depression of the phagocytic index; the percent of bacteria ingested is some 40% less than in normal plasma, and the percent of cells containing bacteria is some 30% less than in normal plasma. Shock plasma also reduces the bacteriostatic power of the phagocytes. The granulocyte in this respect appears to be damaged more severely than the macrophage. The extent of the injury produced by the plasma from rabbits in advanced shock appears to be no greater than that produced by the plasma of rabbits after two hours of shock, *i.e.* while they are still responsive to transfusion. The injurious property of plasma of the reversibly shocked rabbit persists, with undiminished potency, for at least four hours after transfusion. 3) It is concluded that a leucotoxin develops in the blood of the rabbit in hemorrhagic shock, and that this leucotoxin severely impairs the antibacterial potential of the animal.

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Received September 7, 1956. P.S.E.B.M., 1956, v93.

Reduction of Androstane-3,17-dione by Liver Homogenates.* (22795)

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Androstane-3, 17-dione is formed *in vitro* from androstane-3α, 17β-diol by homogenates of the liver and kidney of the rat, guinea pig and rabbit(1), from 4-androstene-3, 17-dione by guinea pig liver and kidney homogenates (2) and from androsterone and epiandrosterone by guinea pig liver homogenates(3). Furthermore, androstane-3, 17-dione has been postulated as the precursor of urinary androsterone and epiandrosterone(4). It would appear that androstane-3, 17-dione occupies a key position in the intermediary metabolism of androgens. Therefore, the metabolism of this substance by liver homogenates has been investigated.

Methods and procedures. Purification of androstane-3, 17-dione.† The material was submitted to adsorption chromatography on alumina (washed Harshaw). Those fractions which showed only androstane-3, 17-dione on paper chromatography (0.5 mg/spot) were pooled, treated with Darco charcoal, and recrystallized from acetone and water until a constant melting point was obtained. The purity of the material used in most of the experiments was tested further by infra-red analysis.‡ *DPNH and FDP.*§ The DPNH

* This investigation was supported by contract with the U.S. Atomic Energy Commission and by grant from National Cancer Institute of U.S.P.H.S.

† The steroids were generously provided by Ciba Pharmaceutical Products, Inc., and Syntex, S. A.

‡ Analyses were performed with a Perkin-Elmer, Model 21, Infra-red Spectrophotometer.

§ The following abbreviations are used: DPN, diphosphopyridinenucleotide (Pabst Laboratories); DPNH, reduced DPN; FDP, fructose diphosphate (Nutritional Biochemicals Company); NA, nicotinamide (General Biochemicals, Inc.).

was prepared from DPN by the method of Green and Dewan(5) with some alterations. Reductive conversions of 90% were obtained only when DPN:dithionite ratios of 2:1 were employed. The barium salt of FDP was converted to the sodium salt(6). Livers were obtained from adult male guinea pigs, 6 to 10 months old, which were killed by a blow at the base of the skull and bled. The procedure for incubation and isolation of steroid materials was as previously described(7) except that the separation of ketonic from non-ketonic materials with Girard's T reagent was omitted. The fractions obtained after chromatography on alumina were pooled in accordance with their elution properties and a 0.5 mg aliquot of the pooled fractions was submitted to qualitative analysis by paper chromatography(8). Fractions consisting of mixtures or containing non-steroidal impurities were submitted to paper chromatography for separation and purification. In the case of the principal metabolites, purification was carried out until satisfactory melting points and infra-red spectra were obtained. In addition, derivatives were prepared. The fractions were assayed by a modified Zimmerman reaction(9).

Results. Metabolites. Androsterone and epiandrosterone were the principal conversion products. Androstan-17β-ol-3-one and androstane-3α, 17β-diol were detected in much smaller amounts by paper chromatography in some of the experiments. The following is an example of the physical properties of the metabolites. The values obtained for the authentic samples are given in parentheses. Androsterone, m. 186-7° (185-6°); mixed, m. 186-8°; acetate, m. 165-7° (166-8°); oxime, m. 213-15° (214-17°). Epiandrosterone, m. 167-73° (174-5°); mixed, m. 165-72°; oxime, m. 183-4° (185-7°); mixed, m. 184-7°. The infra-red spectra were identical with those of the authentic compounds.

Homogenate. The amounts of metabolites formed were never very large, ranging from 0.4 to 1.2% in the case of androsterone, and from 0.8 to 2.5% in the case of epiandrosterone (Table I). No significant variation in the amounts of metabolites formed was observed when the pH was varied from 5.2 to 8.0, or when the length of the incubation period was varied to 2.5 hours. These results indicated that either only a small amount of apoenzyme for the reduction of the 3 and none for the 17 ketone group was present or that the amount of endogenous co-factor was not adequate to provide a substantial reduction. It is known that under similar conditions oxidation of the 17-hydroxyl of testosterone is greatly accelerated by the addition of DPN(10) with an optimum at about 4 mg/g of guinea pig liver (unpublished). The endogenous DPN of guinea pig liver, however, is only about 0.5 mg/g and the DPNH even lower, 0.1-0.2 mg/g(11,12). Therefore, it seemed reasonable to assume that the homogenate (apoenzyme) needed exogenous DPNH to become fully effective.

TABLE I. Reduction of Androstane-3,17-dione by Guinea Pig Liver Homogenates.*

	Exp. No.	Metabolites† And., %	Epi., %	Recovered androstane-dione, %
Boiled controls	3	0	0	86.2 ± 1.3
Experimental	4	.9 ± .2	1.8 ± .4	84.2 ± 2.4

*Adult non-fasted male guinea pigs used. Steroid (100 mg), 12 g liver and 50 ml Krebs-Ringer bicarbonate buffer, pH 7.3 (one exp. at 7.5) mixed in Waring blendor for 2 min. Mixture transferred quantitatively by washing with 50 ml buffer to Fernbach flask. Incubation at 27° for 2.5 hr; 95% O_2–5% CO_2 was the gaseous phase. Values are means and their stand. errors.

† And. = Androsterone; Epi. = Epiandrosterone.

Effect of co-factors. Since DPNH can be formed effectively by liver homogenates from FDP and DPN(6), this procedure was utilized (Table II). DPN alone did not increase the yield of metabolites. The addition of FDP with DPN, however, produced a substantial increase in the production of epiandrosterone but not androsterone. A further increase in the formation of epiandrosterone and also an increase in androsterone was secured by increasing the pH from 5.1 to 6.7. The same results were observed when an atmosphere of nitrogen was used. The omission of nicotinamide, however, prevented the increased production of both metabolites presumably due to a destruction of the nucleo-

TABLE II. Influence of pH, Fructose Diphosphate (FDP), Diphosphopyridine Nucleotide (DPN) and Gaseous Atmosphere in Reduction of Androstane-3,17-dione by Guinea Pig Liver Homogenates.

Exp. No.	FDP, mg*	pH	Metabolites§ And., %	Epi., %	Aa., %	Total recovery, %‡
			Air atmosphere			
1		5.2	1.0	1.8		89.7
1	170	5.2	2.2	5.8		86.6
2	330	5.1	1.1	4.8		91.9
1	170†	6.5	2.1	1.7		96.6
2	250	6.7	6.0	9.0	.4	83.6
			N. atmosphere			
2	250	6.7	5.8	8.9	.3	95.2

* Liver (12 g) from adult non-fasted male guinea pigs and 50 ml 0.2 M Na_2HPO_4 buffer mixed in Waring blendor for 20 sec. Mixture transferred with 50 ml of buffer to Fernbach flask containing 50 mg steroid, 50 mg DPN and 400 mg NA. Incubation at 37° for 1.5 hr. Air was the gaseous phase.

† NA omitted.

‡ Metabolites plus recovered androstanedione. Control in which liver was boiled gave a recovery of 86.1%.

§ And. = Androsterone; Epi. = Epiandrosterone; Aa. = Androstanolone.

tides by the endogenous nucleotidase(13,14).

Effect of pH. Since pH seemed to be a factor in the yield of metabolites, studies were carried out to determine the optimum pH of the reductase when DPNH was used. The maximum formation of epiandrosterone occurred at about pH 6.7 (Fig. 1), and the yield was approximately twice that observed when FDP and DPN were used (Table II) but the amount of androsterone not only was less than that obtained at pH 6.7 with FDP and DPN but also was not affected by the change in pH. Small but significant amounts of androstan-17β-ol-3-one were formed.

Amount of DPNH. The addition of more than 19 mg of DPNH to the nicotinamide fortified liver homogenate at pH 6.6-6.7 produced only a slight further increase in the formation of epiandrosterone and had no further effect on the yield of androsterone (Fig. 2).

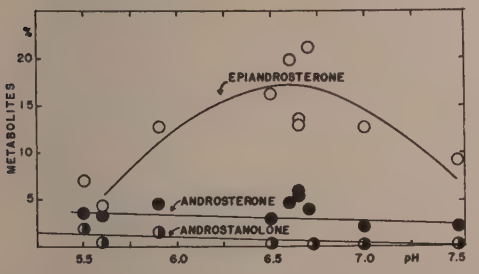

FIG. 1. Effect of pH on reduction of 50 mg of androstane-3,17-dione by 12 g guinea pig liver homogenate in presence of from 37 to 40 mg DPNH and 400 mg NA. Incubation for 1.5 hr at 37° in air atmosphere.

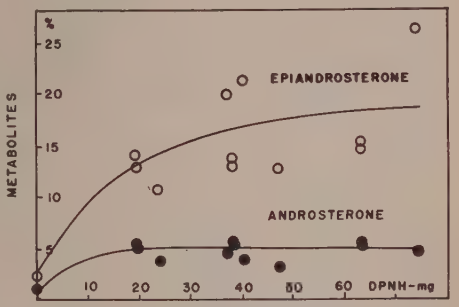

FIG. 2. Effect of quantity of DPNH on reduction of androstane-3,17-dione by guinea pig liver homogenate at pH 6.6-6.7 in presence of 400 mg NA. Incubation was 1.5 hr at 37° in air atmosphere. Total recoveries were from 91-98%. A boiled control yielded no metabolites and 86.1% recovery of androstanedione.

Distribution of androstane-3, 17-dione reductive activity. The liver homogenate was centrifuged at 1500 x g for 5 minutes at 3-8°.‖ The pellet fraction was resuspended in buffer for use. Approximately 75% of the ability to reduce androstane-3, 17-dione remained in the supernate (Table III). The re-

‖ Centrifugations were performed on an International Refrigerated Centrifuge, Model PR-2 provided by the American Cancer Society.

TABLE III. Distribution of Androstanedione Reductive Activity in Adult Male Guinea Pig Liver.

Fraction	Metabolites recovered*			Recovered	Total recovery, %
	And., %	Epi., %	Aa., %	Ae.,* %	
Homogenate	4.6 ± .0	23.0 ± 3.2	trace	57.0 ± .0	84.6 ± 3.2
Supernatant	3.6 ± .8	15.5 ± .1	"	70.5 ± 3.1	89.6 ± 4.0
Sediment	2.7 ± .3	4.7 ± 2.5		88.4 ± .0	95.8 ± 2.8

* And. = Androsterone; Epi. = Epiandrosterone; Aa. = Androstanolone; Ae. = Androstanedione.

Tissue (12 g) and 50 ml 0.2 Na_2HPO_4 buffer (pH 6.6) mixed for 20 sec. in Waring blendor. Centrifugation 5 min. at 3-8° and 1500 × g; supernatants were decanted and sediments resuspended in 50 ml buffer. DPNH added in 50 ml solution. Each flask contained 50 mg steroid and 400 mg NA. Incubation was for 1.5 hr at 37°. Air was the gaseous phase. Results are avg of 2 series of experiments in which 37 and 74 mg of DPNH were used.

mainder of the activity was present in the resuspended pellet. There appeared to be a greater sedimentation of the androsterone-forming activity than of the epiandrosterone-forming activity.

Discussion. The reduction of androstanedione to androsterone and epiandrosterone apparently is accomplished by two different enzyme systems. A similar separation has been demonstrated in preparations of Pseudomonas(15).

Summary. Guinea pig liver homogenates reduce androstane-3, 17-dione to androsterone, epiandrosterone, androstan-17β-ol-3-one and androstane-3α, 17β-diol. The latter two compounds were observed only in trace amounts when addition of co-factors to the incubation mixture produced a marked increase in the amount of metabolites formed. Anaerobic conditions had no effect on the conversion produced by DPN, FDP and Na. The system which produced epiandrosterone exhibited an optimum pH of about 6.7. The formation of the other metabolites, however, was not affected by changes in pH. Approximately three-fourths of the ability to reduce androstane-3, 17-dione was in the supernatant fraction of homogenates centrifuged at 1500 x g.

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Received September 10, 1956. P.S.E.B.M., 1956, v93.

Neuraminic Acid as a Constituent of Human Cerebrospinal Fluid.* (22796)

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Recent investigations on the distribution of neuraminic acid (NA) have revealed that its occurrence in the body is not restricted to its role as a constituent of water-soluble glycolipids of brain(1,2), nor to states of disturbed glycolipid metabolism(1,6), but that NA occurs in a wide variety of body organs and fluids without necessarily constituting an integral unit of a glycolipid molecule(2,3,4,8). Thus, Böhm *et al.*(7,8) have isolated NA from serum, and have shown that normally it is tightly bound to serum proteins, being wholly precipitable with trichloracetic acid. These findings were confirmed by Uzman and Rosen(9) who also established that the bulk of serum NA is bound to alpha-2 globulins, and present in non-dialyzable form though not as a constituent of gangliosides. The NA values for normal serum recorded by Böhm *et al.*(7) was 40-65 mg/100 ml serum. Those recorded by other workers(9) ranged between 44 and 49.6 mg per 100 ml serum. Thus, assuming a normal serum protein of 6g%, the NA would be expected to correspond to approximately 0.67-1.1% of the total serum protein according to Böhm *et al.*(7) and 0.78-0.83% according to Uzman and Rosen(9). If the NA in the cerebrospinal fluid (CSF) were solely derived from serum, the amount of CSF neuraminic acid would be expected to be approximately proportional to the protein content of the CSF, provided that specific protein fractions having a high NA content were not preferentially concentrated in the CSF. It is a well known fact, however, that the bulk of CSF protein consists of albumin, with the globulin fractions constituting a much smaller proportion of the total than in serum(10-12). Evidence for enrichment of specific glycoproteins with high NA content, like the fetuin (6% NA) of Klenk(13) and the mucoprotein (8% NA) of Winzler(13, 14), in human CSF is totally lacking. The extremely low values (2.4 $\pm$ 0.9 μg NA per ml CSF) reported(15) on the basis of analysis of CSF total glycoproteins is further evidence against any selective increase of NA-rich glycoproteins in CSF with respect to serum.

The present investigation was undertaken to establish whether a relationship between the NA and protein content of human CSF did indeed exist, and to ascertain if, in addition, the CSF contained NA in a different state of binding than that found in serum. The latter consideration assumes importance in view of the possibility of release of NA from tissue glycolipids in the course of cerebral metabolism. Our findings demonstrate that human CSF contains 3 to 20 times more NA than would be expected from the CSF protein content, and that only the non-dialyzable NA moiety of the total NA reflects that portion of CSF neuraminic acid derived from serum proteins.

Material. The samples studied represented cerebrospinal fluid obtained by spinal puncture in the course of pneumoencephalography(10-30 ml), myelography (ca. 10 ml) or routine spinal fluid examinations, as well as samples obtained during diagnostic ventriculography (10-15 ml) from unselected cases studied on the Harvard Neurological Unit at the Boston City Hospital. All samples grossly contaminated with blood, or containing more than 5 white cells/cu mm were rejected.

Methods. The determination of neuraminic acid was effected by the method outlined by Böhm *et al.*(7). Total neuraminic acid was usually determined on 3 ml samples of CSF. 3 ml of Bial's reagent was used. This modification did not affect the linearity, if a 3 ml water blank was used, and blank recordings subtracted from the test samples. Duplicates

* This study is part of a project supported by a grant from the U.S.P.H.S., National Institute of Neurological Diseases and Blindness.

checked within ± 1.0%. Non-dialyzable NA was determined by subjecting 3 ml of CSF to dialysis in 27/32 inch viscose tubing overnight. The dialyzed CSF was quantitatively recovered from the dialysis bags (with distilled water washings), made up to a known volume and aliquots taken for NA determination. These were compared with the total NA, and expressed as per cent non-dialyzable NA. Duplicates agreed within ± 2.0%. TCA-precipitable NA was determined by adding 3 ml of 10% TCA to 3 ml of CSF, centrifuging off the ensuing precipitate, and determining the NA content in the precipitate in the manner originally described by Böhm *et al.*(7).

The CSF total protein was determined by the sulfosalicylic acid method of Ayer *et al.* (16) as modified by Merritt and Fremont-Smith(17), inasmuch as this procedure gave the most reliable and reproducible results in the protein concentration ranges involved (duplicates agreed within 1%). The possibility of incomplete precipitation of some glycoproteins is a handicap shared by all other methods of CSF protein determination. In those cases where the partition of neuraminic acid was studied in the serum as well as in the CSF of a subject, the alpha-2 globulins were also determined by paper electrophoresis, using the Spinco-Durrum electrophoresis apparatus, quantitation being effected by use of the Spinco "Analytrol" photometric recording scanner and integrator.

Results. In 49 samples of human CSF examined, the neuraminic acid concentration was found to vary between 10.7 and 33.5 μg per ml. In those samples containing normal amounts of protein (15-30 mg%) the neuraminic acid corresponded in amount to about 10% of the protein (on the average). The concentration of NA did increase with increasing amounts of total protein in the CSF, but there was no proportional relationship. On the other hand, the NA/Total Protein ratio decreased with increasing protein concentration, so that in samples with a total protein above 500 mg%, the value of NA as per cent of total protein approached 1.0%, which is close to the average value in serum (0.8%).

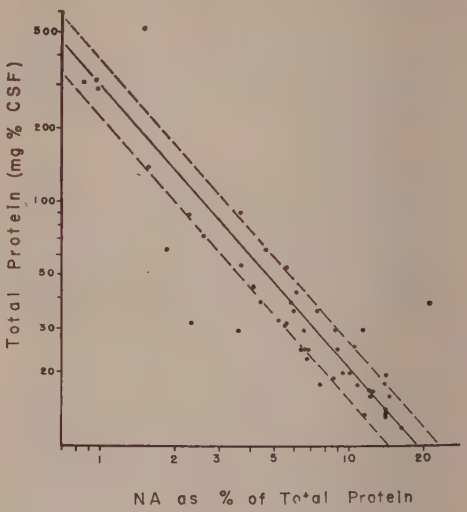

FIG. 1. Showing variation of NA-protein relationship in CSF with increasing conc. of CSF protein.

Thus, although some of the CSF neuraminic acid content is dependent on the NA accounted for by serum proteins, this constitutes a minor portion. Fig. 1 represents the relationship of the CSF neuraminic acid expressed as per cent of total protein to the CSF total protein concentration. It is evident that NA as % total protein decreases with increased protein concentration and rises with decreased protein concentration. Furthermore, this relationship is an exponential one both with the ordinate as well as the abscissa of the graph, so that the majority of values fall along a straight line when plotted against logarithmic ordinate and abscissa. The 5 exceptions represent gross deviations, presumably depending on the pathology presented by the patients involved. In CSF samples with very low protein content (6-10 mg%) obtained by ventricular drainage the NA was proportionately much greater, so that it represented 20-30% of the total protein (top 3 points on Fig. 2).

In those samples where the non-dialyzable NA was estimated, the results indicated that it is this fraction which is derived from serum. In Fig. 2, the log of the non-dialyzable NA, as per cent of the total NA, is plotted against the log of total neuraminic acid as per cent of the total protein. It is evident that as the

TABLE I.

Serum alpha-2 globulins as % total protein	CSF total protein, mg %	NA as % of total protein		Non-dialyzable NA as % total NA		TCA-precipitated NA as % total NA	
		Serum	CSF	Serum	CSF	Serum	CSF
12.8	62	.82	4.6		40.0	95.0	
11.4	13	.81	14.4		21.0	92.4	
12.6	43	.63	6.12	96.5	31.0	88.0	17.85
11.2	64	.49	1.84	100.0	43.0	88.5	22.9
12.1	56	.63	3.66	100.0	43.0	88.0	
9.8	20	.84	9.35	99.5	23.8	92.0	11.7

non-dialyzable NA fraction increases and approaches 100%, the NA/protein ratio drops to levels approaching those prevalent in serum since the NA value expressed as per cent of total protein approaches the serum value of 1.0%. Similarly, low values for per cent non-dialyzable NA correspond to those samples that had low total protein and therefore a high NA/protein ratio.

It is also evident that the relationship of non-dialyzable neuraminic acid to the NA/protein ratio is an exponential one, inasmuch as the values (excepting those marked with arrows), plotted in Fig. 2 fall along a straight line if logarithmic ordinate and abscissa are used. The 4 values, indicated by arrows, that deviate grossly from the straight-line trend represent an extreme type of pathological deviation, the significance of which remains to be elucidated.

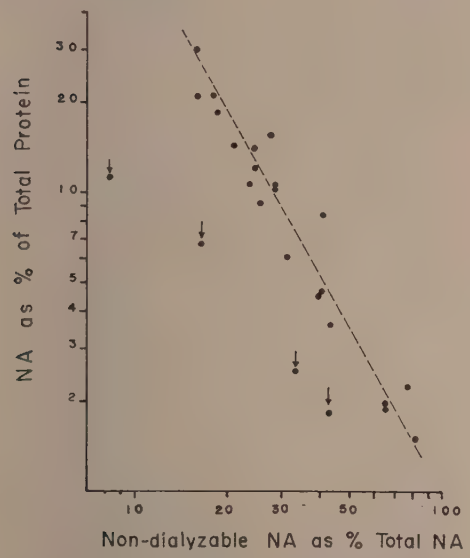

FIG. 2. Variation of non-dialyzable NA moiety of CSF with changes in total NA/protein ratio. Dotted line represents theoretical curve that would be obtained if the whole non-dialyzable NA fraction of the CSF were derived from serum.

That the variations in the NA/protein ratio and the per cent of non-dialyzable NA fraction in the CSF are not due to variations of these values also in the serum of any individual subject is demonstrated by the comparison of values for serum and CSF in 6 randomly selected subjects (Table I). It is apparent that the different state of binding and different NA/protein ratio in the CSF cannot be explained by the NA/protein ratio of the serum. Nor was there any significant difference in the serum alpha-2 globulin concentrations to account for the elevated NA/protein ratios in the CSF. Similarly, the different proportions of the nondialyzable NA fraction in the CSF in contrast to the serum could not be explained on the basis of differences in the alpha-2 globulin content of the sera of the same subjects.

Discussion. From the foregoing information it appears that human cerebrospinal fluid is notable for the high proportion of dialyzable neuraminic acid it contains with respect to its total NA content. The disparity between the dialyzable and bound neuraminic acid in CSF on one hand, and serum on the other, most probably is explained by considerations involving a 2-fold origin for the CSF neuraminic acid. One of these, accounting for the non-dialyzable neuraminic acid fraction, is probably derived from the serum neuraminic acid carried into the CSF by serum proteins. The relationship of the non-dialyzable fraction to the CSF total neuraminic acid with increasing protein levels (NA as per cent of total pro-

tein) as indicated in Fig. 2, clearly supports this view. The second source of neuraminic acid could be the metabolic processes of the central nervous system itself which, presumably in the course of ganglioside turnover, may contribute to the dialyzable, and probably free, neuraminic acid moiety. The fact that it is the dialyzable fraction that is responsible for the disproportionate elevation of NA with respect to protein in CSF clearly argues against the high NA/protein ratio of CSF as being due to selective entry of specific glycoproteins with high NA content. Admittedly, none of the samples studied can be assumed to represent entirely normal values for human CSF, inasmuch as there is always suspicion, if not evidence, of neurological disease when a spinal or ventricular puncture is performed. This fact had, however, the advantage of providing a survey of variations of neuraminic acid content with different levels of CSF protein, and establishing the relationship of the non-dialyzable fraction to the serum-derived neuraminic acid, as shown by our data in Figs. 1 and 2. A further advantage would be evinced in the correlation of neurological pathophysiology and gross deviations from the straight-line trend depicted in Fig. 1. Any increase in NA concentration due solely to increase in CSF total protein would be predicatable within the margin of variation indicated by the dotted lines† in Fig. 1 by the relationship

$$\log (TP) = -1.16 \log (NA) + 2.4771$$

which is the equation for the solid line, where (TP) represents total protein in mg% CSF, and (NA) stands for NA as per cent of total protein. Gross deviations from this numerical relationship, plotted in the same manner as in Fig. 1, would indicate a decreased amount of dialyzable NA if the points fall to the left of the line, and an increased amount of dialyzable NA, if the points fall markedly to the right of the line. It is hoped that extensive correlative studies between the gross deviations of the neuraminic acid concentration from those values expected from the total protein content and the neurological conditions giving rise to these states will prove fruitful in defining the source for the free (or dialyzable) moiety of neuraminic acid in the CSF.

Conclusions. 1. The neuraminic acid (NA) content of 49 samples of human cerebrospinal fluid (CSF) was studied. In all instances the quantitative relationship of neuraminic acid to the cerebrospinal fluid protein was far in excess of that prevalent in serum. In 26 samples in which the total protein was 30 mg % or less, the neuraminic acid was 4-18 times in excess of that expected if it were derived from serum and bound to protein, as is the case in blood. Unlike its state in serum, most of the CSF neuraminic acid is in freely dialyzable form (60-80%). That moiety of CSF neuraminic acid which is derived from serum is in protein-bound, non-dialyzable form. 2. The origin of the dialyzable NA is unknown, but is not related to ingress of serum proteins into the CSF. The implications of these findings are discussed.

† Arbitrarily chosen to include 80% of values. The normal spread is obviously a function of two variables, *i.e.* CSF protein concentration and dialyzable NA, and can only be determined by a study of a very much larger case population.

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Received September 10, 1956. P.S.E.B.M., 1956, v93.

Effect of Dietary Fat on Requirement of Vitamin B_{12} by the Chick.* (22797)

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The young chick's dietary requirement for vit. B_{12} may be influenced by several factors. That the hen's diet affects the vit. B_{12} requirement of her progeny was recognized even before isolation of the vitamin. The requirement of chicks from hens depleted of their vit. B_{12} stores has been shown by most workers to fall between 15 and 30 γ/kg diet(1-4). The vit. B_{12} requirement of chicks from hens fed typical breeder diets was determined by Davis and Briggs(5) to be 1.5 to 2 γ/kg of diet. Similarly, Miller, Norris, and Heuser (6) found that chicks with moderate stores of vit. B_{12} upon hatching required 1.25 γ/kg diet. These values are somewhat below 8 γ, the level that has been tentatively recommended by the Committee on Animal Nutrition of the National Research Council(7). The relationship between vit. B_{12} intake of the hen and requirement of the chick has been quantitated by Milligan, Arscott, and Combs (8). They found that chicks fed a basal corn-soybean oil meal diet (25% protein) require 27, 12, 3, and 0 γ vit. B_{12}/kg diet when the dam's diet contained 0, 4, 8, and 16 γ per kg, respectively.

The influence that certain nutrients of the chick's diet may have upon the requirement of vit. B_{12} has been reviewed by Smith(9). Spivey, Briggs, and Ortiz(10) reported that a severe vit. B_{12} deficiency could be produced in undepleted chicks by the addition of 20% fat to a corn-soybean oil meal diet. The present paper deals with the increase in the vit. B_{12} requirement of chicks receiving this high fat diet.

Methods. The chicks were from a commercial hatchery. They were distributed into groups of 6 chicks each when 1 day old and were kept on the experimental diets for a 4-week period. The chicks were maintained in standard electrically heated batteries with screen wire floors. Feed and water were supplied *ad libitum* and total amount of diet consumed by each group during the experiment was recorded. The chicks were weighed at weekly intervals, and the final weight was the chief criterion of response used in evaluating the results of the experiments. The diets were similar to those used previously(10,11). Diet C29 had the following composition/kg: soybean oil meal 350 g, ground yellow corn 575 g, glucose 10 g, corn oil 5 g, chick salts A 60 g(12), riboflavin 8 mg, vit. D_3 0.02 mg, and 2-methyl-1, 4-naphthoquinone 1 mg. The small amounts of glucose and corn oil served as vehicles for the vitamins. Diet C30 was identical with diet C29 except that 200 g of lard were substituted for an equivalent amount of corn in a kg of diet. The total fat content of diet C29 was approximately 3%, by calculation, and that of C30, 22%. The vit. B_{12} content of each diet was varied in these experiments at levels between 5 and 2,000 γ/kg of diet. For parenteral administration, an aqueous solution of the vitamin was injected subcutaneously in the abdomen

* Part of these data was presented at 39th Meeting of Fed. of Am. Soc. for Exp. Biol., San Francisco, Apr. 11, 1955.

TABLE I. Effect of Dietary Fat on Growth of Chicks Fed Varying Levels of Vit. B_{12}.

Vit. B_{12} in diet (γ/kg)	Diet C29 (3% fat)		Diet C30 (22% fat)	
	No. sig. experiments/ total No. experiments*	4-wk wt (g)	No. sig. experiments/ total No. experiments*	4-wk wt (g)
0	/6	274 ± 7‡	/12	162 ± 5‡
5	1/2	334 ± 13	0/ 6	191 ± 9
5, inj.†	1/1	318 ± 12	2/ 3	226 ± 10
10	1/1	363 ± 19	7/ 8	259 ± 7
10, inj.†	—	—	3/ 3	257 ± 10
20	1/2	321 ± 11	3/ 3	269 ± 12
50	—	—	4/ 5	282 ± 10
100	3/6	343 ± 7	12/12	292 ± 5
200	—	—	3/ 3	291 ± 14
2000	0/1	326 ± 13	3/ 3	302 ± 8

* No. of experiments in which growth was significantly greater (at 1 or 5% level of probability) than that of the control group receiving no vit. B_{12}. There were 6 chicks per experimental group.

† Vit. B_{12} inj. 3 times weekly in the same quantity as that eaten by respective control groups.

‡ Mean ± S.E.

3 times weekly. The vitamin was injected in the same quantity as the mean amount consumed in the diet by the control chicks since the time of the previous injection. At the end of the 4-week experiments, the chicks were killed by decapitation, the livers were removed, and pooled by groups for vit. B_{12} assay. The analyses were carried out according to the USP XV procedure(13) using *Lactobacillus leichmannii*.

Results. The effect of graded levels of vit. B_{12} upon growth of chicks fed either of the 2 diets may be seen from the data presented in Table I. With diet C29, 5γ vit. B_{12}/kg of diet promoted optimal growth in all tests. In some experiments, the chicks grew maximally even in the absence of dietary vit. B_{12}, so the requirement with this diet always fell between 0 and 5 γ vit. B_{12}.

With diet C30, the growth responses to various levels of vit. B_{12} were markedly different from those with diet C29. As reported previously, growth with this diet was always poor in the absence of vit. B_{12}(10). The 5 γ level failed to improve growth significantly in 6 experiments. With increasing additions of vit. B_{12} up to 50 and 100 γ/kg of diet, growth progressively improved to a maximal level. Considerable variation was observed between experiments in the growth response to vit. B_{12}. Although the details are not presented in Table I, 100 γ vit. B_{12}/kg of diet supported growth that was significantly better than that with 50 γ in 3 of 5 experiments where these two levels were fed (average 4-week weight 304 and 261 g, respectively; $p = <0.05$). In the 2 other experiments maximal growth was attained at 4 weeks with 50 γ vit. B_{12}. Addition of vit. B_{12} in quantities greater than 100 γ/kg of diet resulted in no further improvement in growth. The requirement under these conditions, therefore, falls between 50 and 100 γ/kg of diet.

From the growth data in Table I, it appears that with the low level of 5 γ vit. B_{12} in diet C30, the injected chicks grew better than the controls receiving the vitamin in the diet. When only the weights of chicks are considered from the three experiments in which the vitamin was both fed and injected, the chicks receiving 5 γ vit. B_{12}/kg of diet averaged 171 g ± 10 and the injected groups averaged 226 g ± 10 ($p = <0.01$). At the lowest part of the vit. B_{12} growth curve, small amounts of the vitamin resulted in appreciable increments in weight gain; therefore, the slight increase in activity due to injecting the vitamin could be demonstrated at this suboptimal level. With 10 γ vit. B_{12}/kg of diet C30 and 5 γ in diet C29, no differences in growth were noted between groups of chicks receiving the vitamin by mouth or by injection.

The livers from most of the groups represented in Table I were analyzed for vit. B_{12}. For ease of presentation, the values obtained

TABLE II. Storage of Vit. B_{12} in Livers of Chicks Fed Varying Levels of Vit. B_{12} in Low and High Fat Diets (6 Chicks/Group).

Vit. B_{12} in diet (γ/kg)	Liver wt (g)	Vit. B_{12} in liver		Vit. B_{12} intake, avg/ chick/4 wk (γ)	Intake stored in liver (%)
		mγ/g	γ/liver		
Diet C29 (3% fat)					
0	7.6	15	.11	.0	
5	8.2	40	.33	2.95	7
5, inj.*	8.0	50	.40	2.95	10
10	9.0	105	.94	6.35	13
100	8.8	605	5.32	62.40	8
2000	7.3	820	5.98	1108.00	0.5
Diet C30 (22% fat)					
0	3.6	35	.13	.0	
5	5.7	35	.20	1.84	4
5, inj.*	6.4	50	.32	1.84	10
10	7.0	70	.49	5.00	7
50	6.5	160	1.04	19.80	5
75	6.8	265	1.80	31.72	5
100	5.7	440	2.51	42.20	6
200	6.8	540	3.67	888.00	0.4
2000	7.6	780	5.93	912.00	0.6

* Vit. B_{12} inj. 3 times weekly in the same quantity as that eaten by respective control groups.

in one experiment only are presented in Table II. The changes demonstrated by these data are representative of all experiments. In the absence of dietary vit. B_{12}, the amount of vitamin in the liver was essentially the same for the 2 diets. Also, with each diet, increasing levels of vit. B_{12} in the diet resulted in increased concentrations of the vitamin in the liver. This progressive increase in liver stores was more pronounced with diet C29 than with diet C30, because the average 4-week consumption of vit. B_{12} was slightly higher with diet C29 at each level of dietary vit. B_{12}. The proportion of the intake retained in the liver was essentially the same up to the 100 γ level with each diet. In general this percentage was slightly lower with diet C30 than with diet C29. With both diets, the extremely high level of 2 mg vit. B_{12}/kg of diet resulted in very high concentrations in the liver, but this represented a very small proportion of the intake. Similarly, 200 γ/kg of diet C30 resulted in a low rate of storage. The injected vitamin appeared to be stored slightly better than that fed in the diet.

Discussion. Incorporation of 20% fat into a corn-soybean oil meal diet not only increases the severity of a vit. B_{12} deficiency in non-depleted chicks but also increases the requirement for vit. B_{12}. This increase is 10 to 20 times the requirement of control chicks fed the low fat diet (3%) and about 2 to 4 times the requirement reported by others for depleted chicks. The high level of fat did not result in depletion of the animal's chief store of the vitamin, since the content of vit. B_{12} in the liver was similar for chicks fed the high and the low fat diets with no dietary source of vit. B_{12} for 4 weeks. Also, the fat did not prevent the normal absorption of vit. B_{12} from the intestinal tract and its subsequent storage in the liver.

Since vit. B_{12} becomes a critically limiting nutrient when the fat content of this diet is increased, these studies suggest a possible role for vit. B_{12} in the utilization of dietary fat. However, the chicks fed diet C30 did not have any apparent difficulty in metabolizing the large amount of dietary fat, even in the absence of vit. B_{12}. All livers appeared normal upon gross observation; the concentrations of water, total lipid, and phospholipid of the livers fell within normal ranges (unpublished data). Extraction of the feces from chicks fed this diet indicated almost complete absorption of the fat in the diet.

It is possible that the effect of fat upon the chick's requirement for vit. B_{12} is indirect and may be mediated through some other nutrient.

The substitution of 20% lard for an equivalent amount of corn increased the caloric density of the diet by approximately 40% and the ratio of calories to dietary protein by about 50%. The carbohydrate content of the diet was similarly reduced. These altered relationships may have been involved in the increased requirement for vit. B_{12} observed with the high fat diet.

Further discussion of the mechanism by which the high fat diet increased the chick's vit. B_{12} requirement will be considered more appropriately in a subsequent report on the "sparing" effect of certain nutrients upon vit. B_{12} under these conditions.

The graded growth response to varying levels of vit. B_{12} which has been obtained, makes diet C30 suitable for assessing the vit. B_{12} activity of concentrates or analogues for the chick. This diet has proven very useful in our laboratory for this purpose(11). The addition of other nutrients, such as methionine or choline, may alter the chick's response to vit. B_{12}, therefore, this diet cannot be used to evaluate the vit. B_{12} activity of low potency materials.

Summary. Incorporation of 20% fat into a corn-soybean oil meal diet increased ten to twenty-fold the vit. B_{12} requirement of non-depleted chicks for optimal growth at 4 weeks of age. The requirement was between 0 and 5 γ per kg of diet (3% fat) and between 50 and 100 γ with the high fat diet. The high dietary fat did not deplete the chick of its vit. B_{12} liver store or alter the absorption of dietary vit. B_{12} and its subsequent storage in the liver.

The authors gratefully acknowledge the technical assistance of Clarence E. Emery, Helen M. Hood, and Woodrow W. Duvall.

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Received September 12, 1956. P.S.E.B.M., 1956, v93.

Effects of Hypoxia on Iron Absorption in Rats. (22798)

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Many studies have shown that gastro-intestinal absorption of iron is increased, in experimental animals(1,2,3) and human subjects(4), during many diverse types of anemia. Granick(5) has suggested that accompanying hypoxia is probably the only common factor capable of increasing iron absorption.

The present experiments were designed to test the hypothesis that hypoxia is capable of increasing iron absorption without the complicating presence of anemia or dietary changes.

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Methods and materials. We used young male rats of the August strain, each weighing about 120 g. The animals were fed exclusively on M.R.C. diet No. 41,† food and water being freely allowed. During experiments the animals were housed singly in metabolism cages. For the hypoxia experiments the cages were placed in an airtight glass tank through which a gas mixture, consisting of 15% oxygen and 85% nitrogen, was passed at 3 liters per minute. In the corresponding control experiment air was passed through the tank at the same rate. Radioactive iron (Fe^{59}) was administered by stomach tube while the animal was lightly anaesthetized with ether. The amount of iron administered to each rat was 0.6 μc $Fe^{59}Cl_3$ (less than 1 μg Fe) contained in 0.2 ml acetate buffer at pH 5. The following experiments were performed: 1. Controls: 8 animals were exposed to air at 3 liters flow/minute for 48 hours, then given Fe^{59} and immediately replaced in the same environment for a further 24 hours. 2. 9 animals were exposed to the hypoxic atmosphere for 48 hours, then given Fe^{59} and immediately replaced in the hypoxic atmosphere for a further 24 hours. In the remaining group, animals were kept at normal room atmosphere. 3. 27 control animals were given only Fe^{59}. In each case, feces were collected daily for 6 days. Feces were placed in small glass tubes and their radioactivity assayed by direct counting in a well-type scintillation counter (efficiency for Fe^{59} approximately 10%). Fe^{59} was administered at approximately the same time of day in each experiment, and it was noted that food intake and volume of fecal excretion were normal during experiments.

Results. The results of the 3 experiments are summarized in Table I. There was no significant difference between iron absorption in free air and in the glass tank at 3 liters/minute. However, in Exp. 2, where animals were exposed to 48 hours of hypoxia prior to Fe^{59} administration, there was a significant increase in iron absorption by average of 31%. ($P<0.001$).

† Medical Research Council diet No. 41 is a balanced diet with adequate mineral content.

TABLE I. Fe^{59} Absorption in Control and Hypoxic Rats.

	No. of animals	Mean absorption, % of administered dose	Range, %
1. Control: air, 3 l/min.	8	24 ± 2.83*	16-40
2. Hypoxia (15% O_2), 3 l/min.	9	55 ± 5.0	37-76
3. Control: free air	27	33 ± 1.54	16-47

* Stand. error of mean.

Throughout these experiments it was found that Fe^{59} excretion was maximal throughout the first 24 hours and thereafter fell off rapidly to negligible amounts after 6 days.

Discussion. These results confirm the hypothesis that hypoxia is capable of increasing absorption of iron from the gastro-intestinal tract without the presence of anemia or dietary changes.

The mechanism by which hypoxia increases gastro-intestinal absorption of iron remains obscure. However, recently it has been shown that hypoxia causes a redistribution of tissue iron(6) and an increase in chromoprotein oxygen transport systems(7,8). Possibly iron is mobilized from the intestinal mucosa for this purpose, under the influence of hypoxic stress; however, these processes probably occur too slowly to account for our results. On the other hand, Granick(9), in a recent review of iron metabolism, has suggested that hypoxia may influence iron absorption by an indirect effect on a humoral mechanism influencing the intestinal mucosa. Undoubtedly, hypoxia is capable of influencing iron metabolism very rapidly. In this respect, the studies of Huff and co-workers(10) are of interest. They found that plasma iron turnover rates and red cell iron renewal rates were increased to approximately twice their normal values after less than 12 hours at high altitude.

Another explanation of these findings is that the hypoxic stimulation of erythropoiesis sets off a humoral mechanism which is capable of causing increased gastro-intestinal iron absorption.

Summary. 1. Experiments with Fe^{59} were carried out to test the hypothesis that hypoxia

increases iron absorption. 2. Hypoxia was found to increase iron absorption by approximately one-third.

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Received September 13, 1956. P.S.E.B.M., 1956, v93.

A Simple Method of Cutting Split Thickness Skin Grafts from Small Animals. (22799)

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The great increase in interest in homografting and especially in that of skin homografting prompts us to report what we have found to be a simple and reliable method of obtaining split thickness grafts from the rabbit and rat. Full thickness, pinch, and split thickness grafts all have their advocates and peculiar advantages. The split thickness graft, however, has been especially useful in our work for several reasons. 1) It has a high initial take (we have not yet had a failure). 2) Its hairlessness, softness and thinness make viability easy to evaluate, and also make it ideal for use in millipore chamber technics. 3) It requires no dressing of any kind.

Methods heretofore mentioned in the literature are either too cumbersome or require expensive equipment such as the Brown Electro dermatome. We have found the following method to be simple, rapid, and dependable. The equipment required is inexpensive and can be used by relatively inexperienced persons.

Method. In essence the skin to be used is stretched tightly over a flat block by means of a special clamp and a graft is cut freehanded.

In detail: 1) Donors are clipped closely and widely over the donor site and the remaining stubble is epilated with Barium Sulphide and corn starch paste. The donors are then kept for 1-3 days at which time a fine stubble of new hair growth will indicate the anlagen phase of the skin or hair growth cycle. 2) Under barbiturate anesthesia an incision is made at one end of the donor area and the skin and panniculus carnosa is elevated from the underlying fascia by blunt dissection. 3) The specially shaped block (Fig. 1) is introduced into the space thus formed and the clamp is applied so as to hold the skin stretched tightly over the block. 4) At this point it is advantageous to make any desired identifying marks by pricking the skin with a #24 needle dipped in India ink (Fig. 2). 5) The graft can now be cut easily with a straight razor held freehand. The clamp serves as a handle to steady the skin. Grafts of as much as 60 sq cm can be cut in a single sheet from a 300 g rat (Fig. 2). 6) The clamp is removed, the block withdrawn and the incision closed with Michel clips or may be sutured as desired. 7) The grafts may be applied to the recipient bed by any method. We have found Michel clips to be a very rapid and entirely satisfactory way of securing the graft (Fig. 3). 8) By placing the

FIG. 1. Stainless steel clamp and three Duraluminum blocks of different widths which it will fit.

grafts on the dorsum of the recipients and isolating the animals for the first 48 hours we have not found it necessary to use any type of protective or pressure dressing.

In 25 rats autografted with 4 x 10 cm grafts the only slough which occurred was in the small points of pressure from the clips. Otherwise, all grafts could be considered 100% takes. Infection or hematoma formation under the grafts has not occurred at any time even in animals severely debilitated by toxic medications. No attempt at sterile technic has been made.

Summary. The details of a new method of cutting split thickness skin grafts from small animals are reported with a discussion of some of the advantages.

Received September 17, 1956. P.S.E.B.M., 1956, v93.

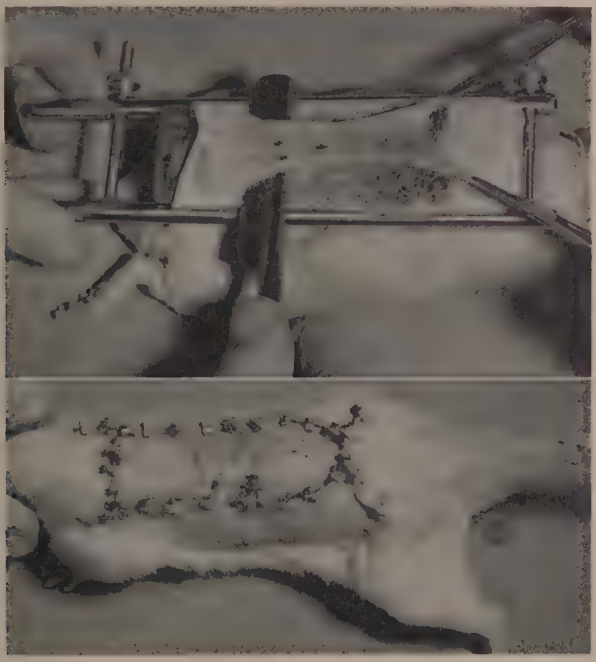

FIG. 2 (top). Clamp in place and graft being cut. Notice identifying tattoos.
FIG. 3 (bottom). Homograft on 6th postoperative day. No dressing was used. The granulating area represents a button hole in the graft. The dark mottling in the center of the graft was reddish-purple at the time of the photograph, and was the first area of slough.

Effect of Extracts of Developing Muscle on Tissue Culture.* (22800)

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Gaillard(1) has shown that the growth promoting activity of extracts of whole chick embryos varies depending upon the age of the extracted embryos. From these experiments little could be concluded as to a possible correlation of growth promoting activity and the state of proliferation and differentiation of the donor tissue since whole embryo extracts include components of tissues at widely differing stages of development. Examination of extracts from adult tissues with different rates of cell proliferation did not indicate that proliferative activity in a tissue and growth promoting activity of its extracts are related (2). As a result of a more systematic study of the cytological and chemical changes in developing leg muscle of the chick(3,4), data have become available which invite a reconsideration of the relationships of the developmental stage in a single tissue and the growth promoting activity of its extracts. The results of tests of the growth promoting activity of extracts from developing chick leg musculature are reported in this paper.

Materials and methods. The muscle extracts to be tested were prepared by carefully dissecting the muscle tissue from the legs and passing it through a Latapie grinder. For each gram of pulp 1 ml of Pannett and Compton (P.C.) solution(5) was added. The mixture was immediately centrifuged (2,000 x g) and the supernatant decanted (designated as 50% extract). The tested extracts were obtained from leg muscle of the 12 day and 19 day chick embryo, and the 10 day and one-year chick because the growth characteristics of muscle tissue differ qualitatively and quantitatively at these stages of development. The mean protein concentration in muscle extracts, determined following Lowry's procedure(6), corresponds to 10.3, 19.7, 20.9 and 25.4 mg protein per ml of 25% extract, respectively for the extracts from the 4 ages given above. The embryo extract used for the first 4 passages was prepared in a similar way using the entire 12 day embryo as starting material. Coverslip cultures derived from leg muscle explants of 12 day embryos were used to assay the growth stimulating activity of muscle extracts. During 4 passages the cells were grown in a clot medium consisting of 2 drops of chicken plasma and 2 drops of 25% embryo extract with transfer to new medium every other day. At the fifth passage the whole embryo extract in the medium was replaced by P. C. solution (controls) or by muscle extracts of the desired concentration and donor age. Twelve hours and 48 hours after the last transfer the surface area of the cultures was recorded with a camera lucida, measured with a planimeter, and the relative increase computed according to Ebeling(7).

Results. The reported measurements of cell culture areas include both cell migration and cell proliferation in unknown proportions. Therefore, the term "growth" can be used in the context of this paper only with the reservation of this incomplete definition. Analysis of the results listed in Table I shows that addition of 0.125% extract from muscle of any of the 4 tested stages of development does not stimulate significantly‡ the "growth" of the test cultures. With extracts from 12 day embryos maximal activity is obtained only with the highest concentration tested (12.5%) whereas the extracts from muscle of the 12 day embryo and the 10 day chick give the same maximal "growth" stimulation with a 10 times lower concentration. The extracts of the one-year-old chick muscle remain sig-

* This work was supported by grant from Muscular Dystrophy Assn. and was carried out as partial fulfillment of requirements for degree of Master of Science in Dept. of Biol., Univ., of Colo.

† The authors are indebted to Dr. U. R. Konigsberg for suggestions concerning technics and to Dr. R. L. Sidman for critique of the results.

‡ Significance is attributed to differences with a statistical probability of chance occurrence of less than 1%.

TABLE I. Compiled Results of Clot Culture Experiments.

Age of extract donor	Conc. of extract in medium, %	Mean relative increase,* mm^2	No. of samples
12-day embryo	.125	3.53 ± .5	15
	1.25	6.74 ± .4	47
	12.5	10.40 ± .5	53
19-day embryo	.125	3.27 ± .4	31
	1.25	11.87 ±1.1	29
	12.5	10.90 ± .5	42
10-day hatched chick	.125	5.14 ± .7	17
	1.25	11.80 ±1.0	44
	12.5	8.52 ± .6	53
1-year-old adult	.125	3.48 ± .6	10
	1.25	5.63 ± .4	33
	12.5	7.46 ± .4	27
Controls	.00	4.71 ± .6	25

* $\frac{\text{Area}_2\ (48\text{ hr}) - \text{Area}_1\ (12\text{ hr})}{\text{Area}_1\ (12\text{ hr})}$ ± S.E. of mean.

nificantly below maximal activity even in the highest concentration. The decrease in the "growth" stimulation with the 19 day embryo extract (12.5%) is statistically insignificant, while the drop in the activity of the 10 day chick extract (12.5%) is significant.

Since the 19 day embryo extracts and the 10 day chick extracts give maximum "growth" promoting activity with the 1.25% extract concentration, the accumulation of the "growth" promoting substances apparently reaches an optimum during the last days of the pre-hatching and the first days of the post-hatching periods. Whether the extracts from the 19 day embryo or the 10 day chick are more active can not be conclusively stated at the present time. A reversal in "growth" promoting activity by excessively high concentrations of tissue extracts has been observed repeatedly(1,8) but its significance has been questioned(9) because of the frequently occurring liquefaction of the media. In the present series of experiments, cultures with liquefied plasma clot were not measured and, therefore, the marked decrease in growth stimulation with the highest concentration of 10 day chick extract may indicate a greater excess of "growth" promoting substances than in the muscle extracts from other stages of development.

Discussion. The period from the 12th to the 19th day of development of the chick embryo is a phase of development in which the rate of proliferation of the leg muscle declines. Correspondingly, total accumulation of DNA, as an index of cell proliferation, was found to come practically to a standstill on the 18th day of development(4,10). On the other hand, formation of specific cell proteins such as collagen (fibroblasts)(11) or of myosin(12) (myoblasts) takes place at a rapid rate during the 12th to the 18th day period of development and myosin production continues at a considerable rate for several weeks after this date. It is apparent that a maximal "growth" promoting activity of muscle extracts is observed when cell proliferation declines sharply and formation of specific cell proteins becomes the predominant growth component.

Summary. 1. Determinations were carried out of the effect of extracts from developing chick leg muscle on the increase in area of coverslip cultures derived from chick leg muscle cells. 2. Maximal stimulation with the lowest extract concentration was obtained with extracts from muscle of the 19 day embryo and the 10 day chick. Extracts from the 12 day embryo and the one-year chick were less effective. 3. The activity of the muscle extracts was found to be unrelated to the rate of cell proliferation in chick muscle tissue.

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Received September 18, 1956. P.S.E.B.M., 1956, v93.

Effect of Hypothermia upon Induced Bacteremia.* (22801)

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In this laboratory observations have been made upon dogs maintained hypothermic for prolonged periods of time(1). The cause of death in some of these animals during the period of cooling and following rewarming remains unexplained. In a study by Parkins, Ben, and Vars(2), upon the effect of differential cooling in fatal ischemic shock, it was shown that a greater number of animals survived preferential cooling of the intestine than primary cooling of the liver. This led Rhoads *et al.*(3), to suggest that intestinal bacteria or their endotoxins might be a factor contributing to the poor results obtained from paramount cooling of the liver. Overton and DeBakey observed(4) that anesthesia and hypothermia, with autonomic blocking agents, was followed by a mortality of 10% when antibacterial therapy was not employed. That a bacterial factor exists in hemorrhagic shock in normothermic dogs is indicated by the experiments of Schweinburg, Frank, and Fine(5). Whether such a factor is present in the hypotension of prolonged hypothermia and subsequent rewarming has not been ascertained. The purpose of this paper is to present data showing (1) that hypothermia even after many hours does not promote a state of bacteremia, and (2) that the hypothermic animal is still capable of removing pathogenic organisms injected into its blood stream.

Methods. Healthy mongrel dogs of both sexes weighing 6.8 to 20.4 kg were used. The technic of cooling used in this laboratory has been adequately described in detail(1). Briefly, animals anesthetized with ether, were placed in a cold water bath at 1°C until the rectal temperature was 28°-29°C. Following removal from bath they were maintained at 22°-24°C in air-conditioned room. Rewarming was accomplished by placing dogs in a 40°C water bath where they were kept until rectal temperature was 28°-29°C. Following drying with towels, animals were placed in warm room where they returned to normothermic levels. Blood samples were obtained by percutaneous puncture of jugular vein. Sterile conditions were maintained during periods of sampling and culturing. To determine presence or absence of bacteremia during hypothermia and during rewarming, blood samples were obtained just prior to cooling following 6 or 12 hours of hypothermia and after 2 to 8 hours of rewarming. These samples were then incubated 48 hours at 37°C in brain heart infusion for determination of aerobic growth and chopped meat broth for anaerobic growth. Transfers were made from thioglycolate broth to blood agar plates and Levine E.M.B. plates and observed for growth and identification of organisms. Transfers from chopped meat broth to blood agar plates were incubated in environment of CO_2 at 37°C. The effect of hypothermia and rewarming on bacterial clearance was determined by intravenous injection of saline suspension of 24 hour culture of *E. coli* and *A. aerogenes* which had been prepared previously from cultures of stools of the animals. All dogs were inoculated with 5-50 billion bacteria. The number of organisms introduced was determined by nephelometry. The first sample of blood, 2 cc, was taken 10 minutes after injection and mixed with liquid agar, plated in duplicate and incubated at 37°C. Succeeding samples were drawn at 6, 12, 18, and 24 hours. Following withdrawal of the 24 hour sample, rewarming of the animal was instituted. Additional 2 cc samples were taken and plated 6, 12, and 24 hours after removal of animal from the warm water

* This investigation was supported by grants USPH No. H2065 and Department of Army No. DA-49-007-MD-715.

† John and Mary Markle Scholar.

TABLE I. Blood Clearance of 5-50 Billion Bacteria Injected into 5 Normothermic Dogs.

No. of colonies/ml of blood				
10 min.	6 hr	12 hr	18 hr	24 hr
2,750	9	3	7	0
5,025	0	1	3	0
2,750	23	3	4	0
4,775	5	4	3	0
5,770	4	2	3	0

bath. After a 48 hour incubation period at 37°C, bacterial colony counts were made on a Quebec-Colony Counter. An average of the number of colonies on duplicate plates was made and results expressed as number of colonies per cc of blood.

Results. Twelve animals were cooled either 6 or 12 hours and then rewarmed. All of these animals maintained the sterility of their blood both during the period of hypothermia and following rewarming. In spite of this, 2 of the 6 animals cooled for 12 hours died 5 and 8 hours following rewarming.

To determine the effect of hypothermia and rewarming on the capacity to destroy bacteria, 5 normothermic and 13 hypothermic dogs were inoculated with 5-50 billion bacteria (*E. coli* and *A. aerogenes*). The normothermic dogs (Table I) revealed no ill effects and completely cleared their blood streams of bacteria in 24 hours. Hypothermic animals were divided into 2 groups (Table II). Group I was injected with bacteria when their temperatures were stabilized at 23 ± 0.6°C. Group II was injected 6 hours after hypothermia had been maintained at 23 ± 0.6°C. In the first group all animals except one had positive blood cultures after 24 hours of hypothermia. Following rewarming all animals in this group demonstrated negative blood cultures within 24 hours. Three of the dogs died 12, 42, and 44 hours following rewarming. All of these animals had negative blood cultures before death and at autopsy no apparent cause of death could be ascertained. In the second group, where bacteria had been injected after 6 hours of hypothermia at 23°C the ability to clear organisms appears to be about the same as in the first group. One animal in this group died during hypothermia although a blood culture taken previous to death revealed no growth. Three other dogs died following rewarming. Only one of these demonstrated a bacteremia. In this animal, however, there was a decline from 7000 colonies per cc to 3 colonies per cc during the course of the experiment.

Discussion. Although animals maintained hypothermic for long periods of time did not clear bacteria from the blood as efficiently as the normothermic dogs, phagocytosis was not abolished. Samples of blood from hypothermic dogs, 6 hours subsequent to injection of bacteria, indicate a 99% phagocytosis in all but one animal. In all instances, the 12, 18, and 24 hour samples from cooled animals showed the blood to be free of 99% of the initially injected bacteria. Recently it has

TABLE II. Blood Clearance of 5-50 Billion Bacteria Injected into 2 Groups of Dogs during Hypothermia and after Rewarming.

	No. of colonies/ml of blood								
	During hypothermia					After rewarming			
	10 min.	6 hr	12 hr	18 hr	24 hr	6 hr	12 hr	24 hr	Remarks
Group I	7,900	3	3	7	2	0	0	0	Died
	4,650	2	0	3	0	0	0	0	Survived
	3,775	13	9	6	19	5	4	0	"
	4,450	12	11	1	105	50	5	0	Died
	12,500	4	23	20	14	3	0		"
Group II	2,800	2	0						Died
	9,500	21	50	10	7	3	0		"
	7,000	1,000	27	20	14	7	3		"
	3,000	15	9	3	3	0	0	0	Survived
	9,200	42	60	13	4	2	0	0	"
	8,500	42	13	5	1	0	0	0	"
	8,000	2	2	6	0	0	0		Died

Group I: Bacteria inj. when temp. stabilized at 23.0 ± 0.6°C. Group II: Bacteria inj. 6 hr after hypothermia at 23.0 ± 0.6°C.

been demonstrated that there is an 86% reduction in the leucocyte count in dogs cooled to the range of 18°-26°C for a hundred minutes(6) and that the leukopenia disappears within a half hour of rewarming(7). *In vitro* studies of temperature relations in phagocytosis by Harmon and associates(8) showed that the phagocytic powers of the polymorphonuclear leucocytes decreased with lowered temperatures. Since the experiments in this report encompass the time interval reported by Helmsworth(6), a leukopenia should be expected at the time of the injection of the bacteria. It would thus seem that the paucity of leucocytes and the decline of the phagocytic powers of the remaining leucocytes could not account for the 99% removal of the injected bacteria as seen here. It is reasonable to assume that the reticulo-endothelial system of the liver and spleen assumes the major role in phagocytosis under these conditions. Manwaring(9) and Cannon(10), on a basis of blood flow, availability of macrophages and leucocytes, and mechanical conditions favoring filtration, felt that the liver and spleen were the principal phagocytic centers. Fisher *et al.*(11) showed that there was a progressive decrease in hepatic blood flow as the hours of hypothermia were extended and that on rewarming there was a prompt return to control levels. The removal of bacteria from the circulation as reported here would seem to parallel these changes in blood flow. A trapping and intermittent release of contaminated blood as well as the decrease in hepatic blood flow may account for the incomplete phagocytosis which occurs during the hypothermia. It would seem that it is impossible to relate the cause of death in rewarming following prolonged hypothermia to a bacterial factor or at least a bacteremia. Although 7 out of 12 animals into whose circulation bacteria were injected died after being rewarmed, in only one of these was there a positive blood culture.

Conclusions. 1. Dogs cooled to 23°C 6 or 12 hours and then rewarmed, maintained sterility of blood during cooling and following rewarming, suggesting that the gastrointestinal tract and other bacterial foci maintained their integrity for at least 12 hours of hypothermia. 2. Hypothermic and normothermic animals intravenously inoculated with bacterial suspension were able to rid blood within 6 hours of 99% of the injected pathogens. Within 24 hours after injection of cultures of *A. aerogenes* and *E. coli,* normothermic animals cleared the blood completely of bacteria while dogs maintained hypothermic did not. However, within 12-24 hours after rewarming only 1 of 11 hypothermic animals had a bacteremia. 3. Inability to survive following prolonged hypothermia does not seem to be related to bacteremia.

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Received September 19, 1956. P.S.E.B.M., 1956, v93.

Urinary Excretion of 5-Hydroxyindole Acetic Acid, A Serotonin Metabolite, in Hypertensive Renal-Vascular Disease.* (22802)

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The recent literature has called attention to the syndrome of metastatic carcinoid, manifested by vascular cutaneous flushing, respiratory distress and chronic diarrhea associated with right sided valvular disease of the heart (1-2). The work of Lembeck(3) and Udenfriend(4,6) has resulted in the demonstration of high concentrations of 5-hydroxytryptamine (5HTA, serotonin) in carcinoid tumors and the detection of the 5HTA metabolite, 5-hydroxyindole acetic acid (5HIAA), in urine(5). Sjoerdsma and Udenfriend(6) in their excellent study of patients with the "carcinoid syndrome" have demonstrated high titers of 5HTA in blood and high concentrations of 5HIAA in urine of such patients.

Several authors(2,10) have called attention to the fibrous tissue proliferation present in many of the patients with long standing "carcinoidosis." They have particularly noted the unusual and extensive distribution about the right heart (fibrous thickening of the endocardium with pulmonic and/or tricuspid stenosis) as well as fibrous tissue proliferation in the pelvis and about the abdominal viscera. In some instances evidence of previous inflammatory lesions was noted as indicated by the microscopic description of new blood vessels and occasional round cell infiltration. Because of the vasomotor disturbances, fibrous tissue proliferation and the apparent role of 5HTA metabolism in patients with the "carcinoid syndrome," it seemed worthwhile to study 5HTA's metabolite, 5HIAA in patients demonstrating the severe arterial lesions, which are found in the acute fibrinoid necrosis of malignant hypertension(7,8).

Methods and materials. Twenty-four hour urine specimens were obtained in all instances mentioned in this report with 10 cc of toluene as a preservative. 5HIAA was determined by the procedure of Udenfriend, Titus and Weissbach(5). Normals were obtained on 15 adults and 2 children. Six patients were classified as malignant hypertension because they demonstrated necrotizing arteriolitis and/or papilledema. One of these (J.S., wt. 16 kg) had received Serpasil, 17.80 mg orally in 48 days after which her initial urinary 5HIAA was determined. She then received 19.9 mg of Serpasil I.M. in 8 days with subsequent 5HIAA determinations. Also included are 2 patients presenting the late phase of chronic glomerular nephritis and 2 patients on whom 5HIAA exertion was determined after removal of early carcinoid tumors of the intestinal tract. There has been no reference in the literature concerning the effect of proteinuria on the determination of 5HIAA. The following experiment was performed to rule out the possibility that protein might interfere, since all our patients with hypertension and diminished excretion of 5HIAA had proteinuria. Aliquots of urine from patients with proteinuria (5 to 10 g/day) were analyzed and mixtures were made with known normal urines.

The *results* are summarized in Table I. The average daily excretion of 5HIAA in normal adults was 4.4 mg (ranges 2.3 to 6.3) and for 2 normal children 2.7 and 3.4 mg. Low levels (less than 2.) were found in 3 patients with malignant hypertension and 2 patients with advanced changes of chronic glomerular nephritis. One patient (E.P.) with a markedly elevated blood urea nitrogen (81 mg %) had urinary 5HIAA levels in the normal range. Sjoerdsma(9) and his group call attention to low values in patients with collagen vascular disease (lupus erythematous disseminatus). No mention is made of renal involvement in these patients. Conceivably the low values were in patients with renal vascular involvement. Moreover these investigators confined their description of "low val-

* This study was supported in part by the Hypertensive Foundation, and McCormick & Co., Baltimore, Md.

TABLE I. Urinary Excretion of 5-Hydroxyindole Acetic Acid.

Diagnosis		Comment	No. of patients	Age	Bun. at time of analysis (mg %)	Avg 24 hr urine vol (cc)	Urinary excretion (mg/24 hr)
Normal subjects	Adults		15	18 to 56		1410	2.3 to 6.3, avg 4.4
	Children		2	11		1140	2.7, 3.4
Malignant hypertension	C.L.	Papilledema; autopsy; necrotizing arteriolitis in kidney	1	53	27	1120	1.08
	E.P.	Papilledema	1	40	81	2800	5.4
	E.S.	Papilledema; autopsy; necrotizing arteriolitis of kidney with minimal fibrosis and lymphocytic infiltration	1	29	48	900	2.5
	J.D.	Papilledema; renal biopsy, chronic pyelonephritis with severe arteriolar sclerosis	1	38	38	785	4.8
	G.R.	Papilledema; autopsy, severe arteriolar sclerosis with no inflammatory reaction	1	37	108	200	0.5
	J.S.	Papilledema; autopsy, chronic pyelonephritis with necrotizing arteriolitis	1	7	104	653	1.5 after 17.8 mg Serpasil
					72	797	1.7 } after 1.9 mg
					104	715	0.96 } Serpasil I.M.
Chronic glomerular nephritis	M.S.		1	16	94	1970	1.6
					100	1455	1.9
	W.H.		1	5	88	410	1.4
					20	800	1.2
Carcinoid	E.C.		1	59			2.4, 2.8, 4.0
	S.W.		1	27			3.2

ues" to patients with "collagen vascular disease," while disregarding normal to high levels in such allied collagen disorders as rheumatic fever, rheumatic heart disease and scleroderma (ranges 5.1 to 7.1 mg). Further recording of 5HIAA excretion in patients with proliferative arterial lesions with and without renal involvement will be necessary before any concrete conclusion may be drawn. It must be noted that proteinuric renal disease itself might possibly lead to diminished 5HIAA excretion, although the proteinuria *per se* does not interfere with the determination as indicated by an 82 to 100% recovery of 5HIAA in mixtures of normal urine and the urine from patients with proteinuria.

The reported low values (9) in several cases of malignant disease might also be attributable to concurrent vascular renal disease.

The low excretion of 5HIAA in patients with hypertension might be due either to a poor amine oxidase activity of the diseased kidney, or, more possibly to a retention of serotonin in the blood. These possibilities are presently under investigation in our laboratory.

The 2 patients with early carcinoid tumors excreted normal amounts of 5HIAA, analysis being performed 2-3 weeks after removal of the tumors. Patient S.W. had a small (.5 cm diameter) rectal polyp removed which on microscopic examination revealed a well circumscribed carcinoid tumor. Patient E.C. presented with acute intestinal obstruction, relieved by resection of 45 cm of ileum containing a carcinoid tumor (5 x 6 cm) with metastasis to the accompanying regional lymph nodes. No liver involvement was noted and there were no associated cardio-vascular abnormalities other than fluctuating blood

pressure of 190/110 to 160/100, soft aortic systolic murmur, and electrocardiographic changes suggesting diffuse non-specific myocardial disturbance.

Summary. The urinary excretion of 5-hydroxyindole acetic acid in patients with malignant hypertension and chronic glomerular nephritis is reported. Low levels were recorded for 3 patients exhibiting the malignant phase of hypertensive cardiovascular disease and in each patient with chronic glomerular nephritis. All patients exhibited extensive renal involvement and 5 of the 6 patients with malignant hypertension had severe arterial lesions. The 2 patients on whom determinations were made after removal of early carcinoid tumors did not show abnormal urinary 5-hydroxyindole acetic acid levels. Recovery studies show that proteinuria does not interfere with the determination of 5HIAA in the urine.

We are indebted to Miss Anne Rider for technical assistance.

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Received September 24, 1956. P.S.E.B.M., 1956, v93.

Distribution of C^{14}-Labeled Aminoacetonitrile in Tissues of Rat, Metabolism and Mode of Elimination.* (22803)

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Several recent studies(1,2,3) have dealt with the effects of aminonitriles on rats. The remarkable ability of these substances to produce the characteristic skeletal lesions of lathyrism in young animals suggests the investigation of the metabolism of the aminonitriles, with the aid of the isotopic labeling technic. Very recently isotopically-labeled β-amino-proprionitrile has been reported synthesized and a study of its metabolism begun (4). For the present work, aminoacetonitrile (AAN) labeled with C^{14} in its cyanide group was employed. It has been previously established(1) that this compound resembles closely in its action, but is more potent than the higher homolog, β-aminoproprionitrile, or β-(γ-L-glutamylamino)-proprionitrile, the active principle of Lathyrus peas. Besides determining the distribution of the radioactivity in the animal organism and the modes of elimination, experiments were designed to explore the possible incorporation of the cyanide carbon atom into major body constituents, including protein, lipid, and carbohydrate. One possible route would be via hydrolysis of the cyanide radical to yield a carboxyl group, *i.e.* the transformation of AAN into glycine.

* Aided by U. S. Public Health Grant No. A-149 (C3).

Methods. Synthesis of $(H_2N\text{-}CH_2C^{14}N)_2 H_2SO_4$. The following modification was employed of the method given in Organic Syntheses(5): A solution of NaCN (1 g, 20 mM, containing 1 millicurie of C^{14}) in 1.7 ml water was added slowly to a mixture of 1.1 g of NH_4Cl (20 mM) and 3 ml of 37% formaldehyde (40 mM). When half the NaCN had been

added, 0.76 ml of acetic acid was added at such a rate that both the addition of the remaining cyanide and of the acid ended at the same time. The stirring was continued for 30 minutes, and the methyleneiminoacetonitrile was filtered off; yield, 0.70 g (51.5%). The methyleneiminoacetonitrile was treated with a solution of 1 g of 96% H_2SO_4 in 5 ml of ethanol, and allowed to stand over night. The yield of crystalline aminoacetonitrile hydrogen sulfate was 0.91 g or 43% based on NaCN. The recrystallized product melted at 155-7° with decomposition. Its nitrogen content, 25.9%, was lower than theory (26.65), suggesting that a small proportion of the molecules were in the form: $(H_2NCH_2C^{14}N)H_2SO_4$. Female weanling Sprague-Dawley rats (45-50 g) were given AAN-C^{14} by either: (1) A single IP injection of a neutralized solution of the sulfate salt, or (2) Feeding ground Purina Chow containing 0.01% AAN-C^{14} plus 0.09% non-isotopic AAN for a number of days.† After stated intervals of time, animals were anesthetized, and blood samples taken by heart puncture. The tissues were rapidly removed, weighed, and homogenized with 20 parts of water. Aliquots of each homogenate were dried on steel planchets, and radioactivity measured with a flow-gas Geiger counter. All values were corrected for self-absorption. Subsequently protein was isolated(5) from the tissue homogenates and its C^{14} content determined. In one instance liver protein was hydrolyzed and the radioactivity of its glycine and serine determined(6). Also liver glycogen and lipid were isolated(7) and their radioactivity measured. In other experiments, rats were maintained in metabolic cages for the collection of respiratory CO_2 and urine(8). In studying the forms in which the C^{14} was present in the urine and in the tissues, extensive use was made of the "carrier" method, with 3 to 4 recrystallizations of the isolated substance. In employing this technic, AAN was precipitated by adding alcohol to urine and tissue extracts; urinary urea was isolated by the xanthydrol method(9), creatinine as the picrate(10), and allantoin via the silver and mercury salts(11). In one experiment sodium benzoate was injected together with AAN-C^{14}, and hippuric acid subsequently isolated from the urine(12). Lastly, the procedure of Meyer and Smyth(13) for chondroitin sulfate was employed for the isolation of a polysaccharide fraction‡ from the enlarged epiphyseal cartilage plates of rats maintained on diets containing AAN-C^{14}.

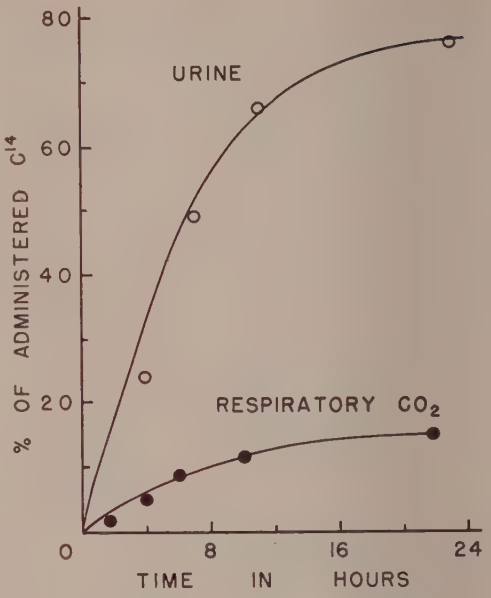

FIG. 1. Rates of elimination of C^{14} in urine and in expired air, following IP inj. of 2 mg AAN-C^{14}. The values are for a group of 6 rats maintained in a common metabolic cage.

Results. Fig. 1 shows that AAN was catabolized to a significant extent, since 15% of the administered C^{14} was found in respiratory CO_2 in 22 hours. Almost 80% of the radioactivity appeared in the urine in this time interval. Further analysis of this same urine, and of urine from rats on a diet containing AAN-C^{14}, revealed that free AAN it-

† Amounts of nitrile are expressed in terms of free AAN, as calculated from quantity of sulfate employed.

‡ This preparation differed from chondroitin sulfuric acid in its lower mobility and more diffuse pattern in paper electrophoresis at pH 8.9. It was sulfated, as indicated by metachromatic staining with toluidine blue. (Kindly performed by Drs. R. D. Montgomery and T. R. Sawyer).

TABLE I. Distribution of Isotope among Urinary Constituents.

Source of urine*	% of C^{14} retained by carrier			
	AAN	Urea	Creatinine	Allantoin
Rats inj. with 2 mg AAN-C^{14}, urine collected first 4 hr.	15	8	3.0	2.1
Same, 4-7 hr urine	18	9	3.5	1.8
Rats on stock diet containing 0.1% AAN-C^{14}, urine collected 6th day	16	12	2.2	1.5

* Pooled urine of 6 rats.

self accounted for only a minor part of the C^{14} eliminated by this route (Table I). The moderate radioactivity in the isolated urea agrees with the finding for respiratory CO_2 The low, but significant isotopic values for creatinine and allantoin suggest that a certain degree of conversion of AAN-C^{14} to glycine-C^{14} occurred, since glycine is a precursor of both these compounds. Further confirmation of this pathway was obtained by injecting a mixture of 1.3 mg AAN-C^{14} and 25 mg sodium benzoate into each of 2 rats. The hippuric acid isolated from the pooled urine (collected over an 18 hour period) accounted for 1.2% of the total administered C^{14}.

Some 60-70% of the C^{14} of urine was not characterized in the present study.§ Schilling and coworkers(4) find that the C^{14} of $NH_2CH_2CH_2C^{14}N$ is likewise chiefly eliminated in the urine of rats, in an altered form. However, they state that the main excretory product "appears to be a volatile, ether-soluble, basic or neutral substance." No volatile products were found in the urine of C^{14}-AAN animals, and only a minor portion of the radioactivity (2-3%) was extractable by ether from either neutral or basic urine. Apparently the 2 nitriles are metabolized differently.

Fig. 2 gives the relative C^{14} levels in body

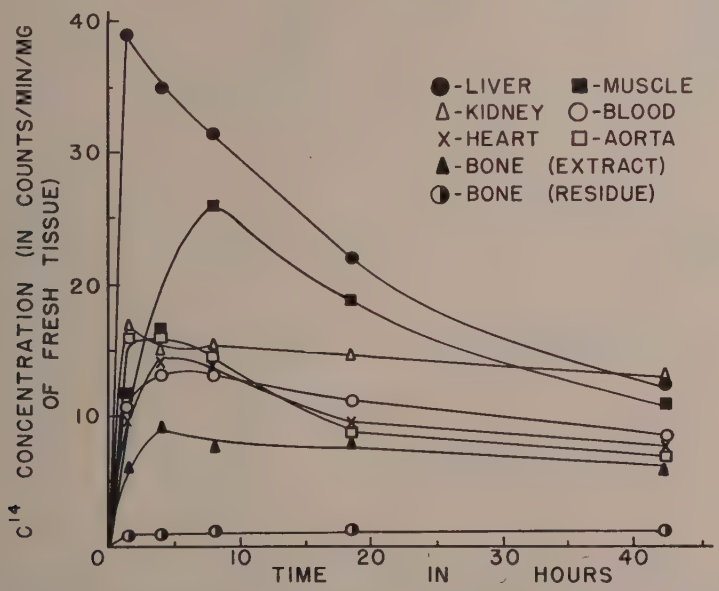

FIG. 2. Distribution of isotope in various tissues, following inj. of 10 mg of AAN-C^{14} per rat. Each point represents pooled material from 3 animals.

§ Attempts to detect C^{14}-labeled thiocyanate by paper chromatography were unsuccessful.

TABLE II. C^{14} Distribution in Rats Fed a Stock Diet Containing 0.1% AAN-C^{14}.

Tissue	C^{14} in cts/min./g fresh tissue		μmoles of labeled substances/g tissue	
	6 days	10 days	6 days	10 days
Liver	7300	7600	2.25	2.34
Kidney	6500	9900	2.00	3.04
Skeletal muscle	2480	3000	.76	.93
Blood	2100	2250	.65	.69
Bone*	2280	2250	.69	.70
Epiphyseal plates of long bones	1770	1990	.55	.61

* Non-mineral extractives.

All values are for pooled tissues of 3 rats. The animals developed moderate lesions at 6 days, and typically severe lesions by 10 days.

tissues at varying times after the administration of a larger quantity of AAN-C^{14} to rats. It may be seen that liver was by far the most active tissue. After 4 hours the specific activity of muscle exceeded that of all other tissues excepting liver, and after about 30 hours, liver, muscle, and kidney were comparable in C^{14} concentration. Blood, heart, aorta, and bone (aqueous extractives) tended toward somewhat lower values, while bone residue was least active. It is evident that the radioactivity was retained by the animals to a greater degree than in Fig. 1, in which the dosage of AAN-C^{14} was smaller.

If the results in Fig. 2 are considered in terms of the total mass of each tissue, it is apparent that skeletal muscle actually accounts for a greater percentage of the isotope than does liver. Likewise, the skeleton, because of its relatively great weight and also its retention of the isotope, contained more C^{14} than any tissue except muscle, and possibly liver.

TABLE III. Isotopic AAN in Rat Tissue.

Tissue	% of C^{14} in each tissue recovered by carrier method		
	Sacrificed 1½ hr after inj. 10 mg AAN-C^{14}	Sacrificed 18 hr after inj. 10 mg AAN-C^{14}	Fed for 10 days diet containing .1% AAN-C^{14}
Blood	73	53	27
Liver	24	16	25
Kidney	33		20
Muscle	61	43	26
Bone	90	58	31
Epiphyseal plates of long bones			39

The animals were those described in Fig. 2 and Table II.

When rats were maintained for a number of days on diets containing AAN-C^{14}, relatively high concentrations of labeled material accumulated in the body tissues (Table II). This is most evident when the radioactivities are converted into μMoles. The values for bone and epiphyseal plates, while not as high as with liver or kidney, do represent substantial accumulations of labeled carbon. Furthermore, it is noteworthy (Table III) that bone was the tissue with the highest percentage of its radioactivity in the form of free AAN; 39% of the C^{14} in the epiphyseal cartilage plates of rats on the AAN-C^{14} diet was present in the form of AAN. These plates were extremely enlarged.

TABLE IV. C^{14} in Tissue Proteins of Rats.

Tissue	Sacrificed 18 hr after inj. 10 mg AAN-C^{14}	Fed 10 days diet containing .1% isotopic AAN
Blood	10	8
Liver	13	12
Kidney		6
Muscle	4	5
Bone	6	7
Epiphyseal plates of long bones		14

The values represent % of total C^{14} in each tissue.

It is of interest that a significant part of the C^{14} in the tissues was in protein combination (Table IV). The high value for epiphyseal plates again reflects the intense synthetic activity occurring in this region. Examination of the liver protein of rats given a single injection of AAN-C^{14} disclosed that 40% of the radioactivity of the protein was due to glycine, while serine accounted for 18%. The distribution of the remaining C^{14} was not determined.

It was found that the sulfated polysaccharide isolated from epiphyseal plates was moderately radioactive (Table V). The position of the labeling was not determined. The C^{14} content of the epiphyseal cartilage protein isolated in this same experiment is given for comparison.

Indication that the C^{14} of AAN partici-

TABLE V. Labeling in Epiphyseal Plate: Rats maintained for 10 days on stock diet containing 0.1% isotopic AAN.

Substance isolated	C^{14} conc./g fresh tissue Cts/min.	μM
Polysaccharide fraction*	95	.029
Protein	157	.048

* Approximately 10 mg were obtained per g of plates.

pated in a variety of reactions was the finding of small but definite concentrations of isotope in glycogen and lipids of liver, 4 hours after injecting AAN-C^{14} into rats. The glycogen accounted for 0.5%, and the lipids 0.6%, of the total C^{14} in this organ.

Summary. Aminoacetonitrile, with C^{14} in its cyanide group, was synthesized. When this compound was administered to weanling rats, the major portion of the radioactivity was eliminated in the urine, while a minor part appeared in respiratory CO_2. High initial C^{14} concentrations were observed in various body tissues. The proportion of this C^{14} due to unchanged nitrile decreased fairly rapidly with time. Labeling was found in tissue proteins, and to a lesser extent in liver lipids and glycogen. Also, in rats fed a diet containing isotopic aminoacetonitrile, radioactivity was detected in the sulfated polysaccharides of the enlarged epiphyseal plates of the bones. The presence of C^{14} in glycine and serine of liver protein and in urinary creatinine, allantoin, and hippuric acid, indicated an appreciable conversion of the cyanide radical into a carboxyl group. However, the major portion of the C^{14}-containing metabolites was not identified.

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Received September 24, 1956. P.S.E.B.M., 1956, v93.

Chronic Effect of Dietary Protein on Hypercholesteremia in the Rat.* (22804)

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A diet consisting of casein, lard and dextrin was found to be atherogenic in the rat(1). Variations in fat and cholesterol content of the diet seemed to have little influence on the consequent coronary arterial lesions(2). A reduction in quality or quantity of dietary protein has raised the serum cholesterol level and promoted atherogenesis in other experimental situations(3,4).

The serum cholesterol level of adult cholesterol-fed rats rises gradually over a period of months to reach a plateau, where it remains for at least another 3 to 6 months. The serum cholesterol after 1 or 2 months may not reflect the ultimate level to be reached when the animal has finally adapted to the diet. On the other hand, the great frequency of a chronic form of pneumonitis(5), which is virtually inescapable in the rat after the age of

* This work was made possible by grants-in-aid from Amer. Heart Assoc. and National Heart Institute of USPHS.

TABLE I. Dietary Variables Expressed as % by Weight.

Series	First				Second			Third	
Group	I	II	III	IV	V	VI	VII	VIII	IX
Casein	18.75	25	12.5	7.5	18.75	9	7.5	40	Purina
Gelatin				10.5					lab
Lard	30	15	15	15	30	15	15	15	chow
Dextrin	32.2	44	56.5	51	32.2	60	61.5	25.4	
Cholesterol	.625	1.2	1.2	1.2	1.2	1.2	1.2	1.2	

The constitution of supplementary salt mix, vitamin mix and other ingredients as well as their sources have been described previously(1,2). All groups received 0.625% choline chloride.

6 months, has led us to limit these studies to a 6-month period.

Method. Male weanling rats of the Sprague-Dawley stock were fed Purina Lab Chow until 3 months of age (250-300 g), when they were divided into dietary groups. They were fed and watered daily and weighed weekly; the food consumption was estimated daily. Each cage contained no more than 8 rats. Under seconal anesthesia and in the fasting state they were bled from the tail vein at 8 weeks, and by cardiac puncture when sacrificed at 25 weeks. Individual serum cholesterol determinations were done in duplicate by the method of Zlatkis(6) and serum phospholipid by a modification of the Fiske-SubbaRow technic(7) on aliquots of a 3:1 alcohol-ether extract of the serum. Gross autopsy of the sacrificed animals was performed, but histological examination was limited to frozen sections of the heart and liver, which were stained with Sudan III. The diets employed were variations from the original diet of Wissler, *et al.*(1), consisting of 30% lard and 18.75% casein. The level of lard was reduced to 15% in all cases, except for this original diet, and the amount of food offered was correspondingly increased. This lower fat level was introduced in order to minimize the incidence of pneumonitis and seemed to make no difference in the ultimate cholesterol level. An increase in level of cholesterol supplement from 0.31 to 0.625 or even 1.2% made no difference in the ultimate hypercholesteremia, though, as others have noted, the higher levels of dietary cholesterol accelerated the rise in serum cholesterol to that level(8). In each case, protein increments replaced dextrin, the fat level remaining constant. In 2 experiments the above pattern was followed in comparing various levels of casein from 25 to 7.5% and a combination of casein and gelatin as outlined in Table I. In a third experiment, using the diet described for group VIII, the rats were 15 months old at the onset of the experimental period, having been on a Purina Chow diet from the date of weaning and they were bled only after 6 months. In this experiment the rats in group VIII were compared with chow-fed animals of the same age. Lipoprotein concentrations of pooled serum of both groups were compared by the technic of Lewis, Green and Page(9) with the analytical ultracentrifuge.

Results. Statistical comparisons (Table II) revealed a level of serum cholesterol in animals fed 12.5% casein for 25 weeks, which was significantly lower ($P<0.01$) than in those fed 25%, 9% or 7.5%. While the cholesterol level attained with 18.75% casein is not enough higher than that reached with 12.5% to achieve statistical significance, it is significantly lower ($P<0.01$) than that resulting from 25% casein or 9% casein in the diet. While it may not be permissible to compare directly the older animals in group VIII with the other groups, their serum cholesterol was so uniquely high for rats on an *ad lib.* diet that they are included here with their chow-fed controls, group IX. No such high levels of serum cholesterol have been observed by us in many other old animals fed the 18.75% casein diet for even longer periods (1,2).

The cholesterol/lipid phosphorus ratios became generally elevated in the first and third experiments where the higher levels of protein were used, and failed to rise significantly at the lower levels of protein fed in the second

TABLE II. Comparison between Mean Serum Cholesterol and Cholesterol/Lipid Phosphorus at Various Levels of Dietary Protein.

Group	No. of rats	Diet: % casein	Diet: % gel	Wt in g: Initial	Wt in g: Final	Wt in g: Gain	Cholesterol (mg %) ± S.E.: 8 wk	Cholesterol (mg %) ± S.E.: 25 wk	Cholesterol/L. phosphorus (mg %) ± S.E.: 8 wk	Cholesterol/L. phosphorus (mg %) ± S.E.: 25 wk
					Exp. 1					
I	18	18.75		285	435	150	121 ± 5.0	158 ± 7.1	24.1 ± .75	25.2 ± 1.07
II	23	25		292	457	165	120 ± 3.0	224 ± 6.6	26.8 ± .70	41.9 ± 1.08
III	22	12.5		290	413	123	148 ± 6.1	148 ± 5.7	25.8 ±1.08	41.3 ± 1.57
IV	23	7.5	10.5	295	354	59	136 ± 3.4	178 ± 6.0	23.9 ± .66	34.9 ± 2.29
					Exp. 2					
V	8	18.75		323	506	183	155 ± 3.8	161 ± 5.6	23.4 ± .4	25.9 ± 1.38
VI	8	9		302	400	88	142 ± 7.9	235 ± 9.1	23.2 ±1.4	27.2 ± 1.40
VII	8	7.5		307	330	23	121 ± 3.0	212 ±29.5	21.4 ± .4	24.4 ± 2.60
					Exp. 3					
VIII	10	40		510	584	74		490 ±75.0		32.5 ± 1.90
IX	9	Chow		482	507	29		162 ±10.2		21.2 ± .82

experiment—even though the serum cholesterol was equally elevated. In the first experiment the terminal ratios are significantly higher in groups II, III and IV than in group I ($P<0.001$), and that of group IV is significantly lower than groups II and III ($P<0.02$). The explanation for a greater disproportion between cholesterol and lipid phosphorus at 25% and 12.5% levels than is seen at 18.75% probably depends upon the fact that the percentage of lard was higher in the latter case(10). In the second experiment the phospholipid tends to rise more nearly parallel to the serum cholesterol, so that even with the higher cholesterol levels no statistically significant differences could be established in the ratios. Again in the third experiment where a very high casein level is fed and a phenomenally high serum cholesterol is seen, the cholesterol/lipid phosphorus ratio is elevated. It appears that the hypercholesteremia of high or adequate casein diets is accompanied by a dramatic increase in the cholesterol/lipid phosphorus ratio, which is not seen with the hypercholesteremia induced by the low levels.

Except for occasional pneumonitis in the older animals of the third experiment, all animals were quite healthy until sacrifice, and gave no evidence of disease on gross autopsy. Histological studies of the heart revealed no coronary artery lesions in any of the groups excepting group VIII where 3 out of 9 animals exhibited subintimal fat staining atheromatous lesions identical to those previously reported in younger rats after one year on the diet fed group I(1,2). Serum lipoprotein concentrations were examined in this group and there was a higher level of faster rising lipoprotein fractions with a lower concentration of high density lipoproteins than in the pooled sera from chow-fed controls (Table III).

Discussion. The serum cholesterol level of the rat is apparently quite sensitive to the sustained protein level in the diet. When gelatin is added to a low casein allowance, hypercholesteremia tends to be less marked (group IV compared to Group VII) though not significantly different from that seen with an equivalent amount of protein derived solely from casein (group I vs. group IV). There is no significant elevation of the cholesterol/lipid phosphorus ratio in the animals on the low casein intake, as compared with those on moderate or higher levels of casein and comparable fat intake; hence, while we may

TABLE III. mg % Lipoproteins Determined on Pooled Sera of 21-Month-old Rats Fed 40% Casein or Chow for Preceding 6 Months.

Diet	−S 1.21* 0–20	20–40	40–70	100–400
40% casein	95	33	65	152
	79	40	57	160
Chow	144	38	15	65
	169	43	27	96

* Flotation rate in 10^{-13} cms/sec. of macromolecules in NaCl-KBr medium of density = 1.21.

postulate an amino acid imbalance in the former case, we would anticipate a different mechanism in the latter.

Fillios and Mann(3) have shown that a hypercholesteremia develops in rats fed a methionine deficient diet, and were able partially to correct the abnormal elevation of serum cholesterol by methionine supplementation. A casein diet of 7½% is suboptimal in methionine and permits the development of a fatty liver which may be partially corrected by further protein or methionine supplement (11).

Higher levels of serum cholesterol have been observed in mice fed high casein diets (80%) than in their litter mate controls fed a low casein (10%) diet(12). Other workers have reported a reduction in hypercholesteremic response with increasing levels of dietary casein up to 60%(13). In this case, however, supplemental cholate was included in the diet, thus masking any choleretic effect of the dietary protein.

The possibility must be considered that it is decrease in carbohydrate rather than increase in protein that is responsible for the hypercholesteremia induced in groups II and VIII. The actual amount of dextrin supplied in each diet is given in Table I, and it can be seen that the percentage supplied in group VIII was almost as much as in groups I and V, so that it seems doubtful that the marked elevation in serum cholesterol in group VIII could be due primarily to the low dextrin level, even though Portman and co-workers (14) have demonstrated a difference between corn starch and simpler sugars in choleretic potential and their effect on experimental hypercholesteremia.

The group of rats fed 40% casein developed a higher cholesterol level than heretofore reported in rats on an *ad lib.* diet free of thiouracil or cholic acid supplements. This correlates with a decrease in alpha lipoprotein and an increase in lower density lipoproteins. The development of coronary atheromatous lesions required a shorter period of time than has been noted previously even in rats of the same age(1,2).

Summary. Chronic dietary experiments in rats have revealed that months are necessary to evaluate the ultimate level of serum lipids resulting from sustained feeding of various levels of dietary protein. Quantitative changes in dietary protein can influence the levels of serum cholesterol and cholesterol/lipid phosphorus ratio. Reduction or elevation of dietary casein beyond a modest range (12-18%) will lead to ultimate elevation of serum cholesterol and phospholipid under conditions herein described. The insignificant change in cholesterol/lipid phosphorus ratio at low levels of dietary casein as opposed to its elevation at higher levels suggests that different mechanisms are operating in these two situations. Unusually high levels of serum cholesterol were achieved in old rats fed 40% casein. Coronary arterial atheromatous lesions developed in one-third of these animals, but in none of the other groups in the 6-month period.

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Received September 27, 1956. P.S.E.B.M., 1956, v93.

Effect on Suckling Young of X-Irradiation of Lactating Mother.* (22805)

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X-irradiation as a general debilitating agent should affect the nursing mouse so that its suckling young would be at least deprived of some nourishment. This study was made to determine the minimum dose level of exposure of the mother which would be reflected in the growth rate of the suckling young.

Materials and method. Adult CF_1 female mice which had had previous litters were used. Only those females with litters of 8 or more were chosen, and each litter in excess of this number was reduced to 8. Two days after delivery the mothers were separated from their litters long enough to be exposed to whole body x-irradiation. The physical facilities consisted of a Quadrocondex constant potential x-ray therapy machine run at 210 KVP and 15 MA, and the filters consisted of 0.5 Al. and 0.28 Cu. The target distance to center of the body of the mouse was 50 cm and output of the machine was 97.7 r/min. in air at that distance. The exposures used ranged from 50 r to 400 r. Immediately following x-irradiation females were returned to their own litters. The simultaneous controls were not exposed to any irradiation. On the day of x-irradiation of the mother, each litter was weighed as a unit. Each litter was weighed three times each week. A total of 70 females with their litters comprised this study. For each dose level used there were at least 8 litters.

It was soon found that suckling normally continued until after day 15 when those mice which were not receiving adequate nourishment turned to the solid food provided for the mother. Therefore, the direct effect on lactation, as reflected in the growth rate, could be determined accurately only until about day 15.

Results and discussion. Data from two separate experiments are given.

Tables from 2 separate sets of data, indicate some effect on growth of suckling young even after 50 r whole body x-irradiation of the mother. There was pronounced retardation of growth where exposure of the mother was 200 r or more. The dose of 400 r, which is closer to the LD/50/30 of 625 r for this strain of mouse, did not stop lactation completely although the suckling young continued to grow at a very slow rate. Retardation of

TABLE I. Average Weights of Young Mice Nursed by X-Irradiated Mothers.

Day	Control	50 r	100 r	200 r	300 r	400 r
3	2.5	2.4	2.4	1.9	2.0	2.3
5	3.8 (.7)	3.5 (1.1)	3.2 (.8)	2.6 (.7)	2.8 (.8)	3.5 (1.2)
8	5.4 (1.6)	5.1 (1.6)	5.3 (2.1)	3.5 (.9)	3.9 (1.1)	4.4 (.9)
10	6.4 (1.0)	5.9 (.8)	6.2 (.9)	4.2 (.7)	4.8 (.9)	4.8 (.4)
12	7.2 (.8)	6.9 (1.0)	6.8 (.6)	4.8 (.6)	5.6 (.8)	4.9 (.1)
15	8.2 (1.0)	7.8 (.9)	7.6 (.8)	5.5 (.7)	5.4 (–.2)	5.1 (.2)

TABLE II.

Day	Control	50 r	100 r	200 r	300 r	400 r
3	2.1	2.0	2.5	2.0	2.1	1.9
5	3.1 (1.0)	2.9 (.9)	3.0 (.5)	3.0 (1.0)	3.0 (.9)	2.7 (.8)
8	4.4 (1.3)	4.3 (1.4)	4.2 (1.2)	4.2 (1.2)	3.9 (.9)	3.6 (.9)
10	5.5 (1.1)	5.1 (.8)	4.9 (.7)	4.8 (.6)	3.9 (.0)	4.4 (.8)
12	6.4 (.9)	5.7 (.6)	5.4 (.5)	5.6 (.8)	4.8 (.9)	5.0 (.6)
15	6.6 (.2)	6.2 (.5)	6.1 (.7)	6.4 (.8)	5.1 (.3)	5.7 (.7)

Note: Increments are in parentheses. Only one is decrement.

* This work was performed under Contract AT-30-1-GEN-70 for Atomic Energy Commission.

† The author acknowledges with pleasure the help of Agatha Nadai.

FIG. 1. Suckling mice at 10 days of age, taken from mothers variously treated on the 3rd day as follows: Left to right—Control, 50 r, 100 r, 200 r, 300 r, 400 r. Note slight stunting due to starvation, progressively greater the greater the irradiation of the mother.

growth of the suckling young appeared to be due to simple starvation and not to passage of toxic substances through the milk. This was demonstrated by replacing the irradiated mothers with normal lactating mothers of the same post-delivery age and determining the effect on the young. Invariably they regained their growth momentum almost immediately so that there could not have been any residual toxic effect. Mice which had been suckling to a 400 r mother for 15 days, when given a normal mother, approached the normal control weight by 30 days of age. True, some of these mice were able to feed on the dry food available to the mother, but the initial recovery of growth momentum was due to the abundance of normal milk.

The reciprocal exchange was made. Mice nursing to normal mothers were, at 15 days, given to previously x-irradiated (200 r or more) mothers. The growth of these mice was abruptly retarded. However, after 3 to 5 days they regained growth momentum under the supplemental influence of self-feeding on dry food. In a few cases young mice from normal mothers were given at 15 days to mothers who had been x-irradiated to 400 r and these were starved to death within 4 to 5 days.

Summary and conclusions. 1. Increasing x-irradiation of nursing female mouse caused increasing retardation of growth of suckling young. There is some effect even after 50 r exposure of the mother on the 3rd day postparturition. There was pronounced effect at 200 r or greater. 2. There is no evidence of any toxic substance passing through the milk to the young. The effect seems to be one of simple starvation, probably through general debilitation of x-irradiated mother. 3. Newborn mice starved by an x-irradiated mother will recover rapidly if given to a normal lactating mother at 15 days postparturition, and by 30 days will be essentially like controls with respect to body weight. Recovery from initial starvation appears to be complete.

Received September 27, 1956. P.S.E.B.M., 1956, v93.

Prolactin and Thyrotrophin Content of Functional Transplantable Pituitary Tumors. (22806)

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Transplantable functional pituitary tumors described recently were classified on the basis of their hormonal secretion as indicated by changes in tumor-bearing hosts(1). Three main types have been studied extensively: adrenotrophic, thyrotrophic and mammary gland stimulating. The adrenocorticotrophin and melanophore stimulating activity of an adrenotrophic tumor(2) and the thyrotrophin (TSH) activity of some thyrotrophic tumors have been determined. The following is a study of prolactin content and a more complete account of the TSH content of various tumors.

Methods. Prolactin was assayed in pigeons by the cropgland weight increase method (3,4). Thyrotrophin was assayed in baby chicks by following the depletion of I^{131} from the thyroid(5). Each powder was a pool of tumors from several mice. All lyophilized or acetone dried tumor powders were put through a Wiley mill using a 60 mesh screen and suspensions of such powders in 0.1 M $NaHCO_3$ solution were injected using a 22 gauge needle. Heparinized blood was collected from abdominal aorta of mice. After centrifuging, the plasma was kept frozen until injected. Prolactin was extracted from 2 tumor powders using 65% ethanol at pH 9.5-10. The alkaline alcoholic extracts were adjusted to the isoelectric region, pH 5-7, and prolactin precipitated in refrigerator by addition of ethanol to a concentration of 85%. This precipitate was washed with acetone and dried. After removal of tumor, adrenals, gonads, pituitary and obvious abdominal metastases, the bodies of 9 radiothyroidectomized mice, which had had large dependent thyrotrophic tumors (Strain 77D) were frozen. Later they were ground in a Waring blendor with 3 volumes of 1% saline and then centrifuged. The cloudy liquid phase was adjusted to pH 4.7 with N/1 HCl and again centrifuged. The clear supernatant at pH 4.7, which should contain any TSH present, was adjusted to pH 7 and lyophilized for bioassay. Seven grams of powder were obtained.

Material. Tumor Strains: Thyrotrophic tumor strain 77D was induced in a C57Bl male mouse by 275 μc of I^{131}, remained dependent for 4 consecutive passages. In one line autonomy was acquired in the fifth subpassage. Thyrotrophic tumor strain 101D was induced in a C57Bl female mouse by 400 μc I^{131}, remained dependent through 2 subtransfers, but one line developed autonomy in third transfer. The present study was made with dependent tumor lines. The origin and characteristics of the mammotrophic tumor strains in mice(6) and rats(7) have been described. Characteristics of the adrenotrophic strain 2 induced by total body ionizing irradiation have been reported(1).

Results. TSH and Prolactin content of tumor powders: There is a very high potency of the 2 dependent TSH tumors (77D and 101D) (Table I). This is about 10 times that of powders from cattle or sheep pituitaries. The other tumors all gave negative tests or borderline responses at the high dose level used. These borderline tests represent concentrations of TSH that are only 1% that found in bovine pituitaries and 0.1% that found in TSH tumors.

Thyrotrophic and adrenotrophic tumors gave negative tests for prolactin. The stilbestrol dependent mammotrophic rat tumor (FR4) was uniformly positive but had only 10% concentration of prolactin found in bovine pituitaries. Powders of mouse mammotrophic tumor (OG4) gave variable results indicating that they had about 10-40% of prolactin potency of the rat tumor (FR4).

* Work supported by research grant C-2259 from National Cancer Inst., P.H.S.

TABLE I. Prolactin and Thyrotrophin Potency of Dried Powder of Six Transplantable Tumors.

Species	Transplantable pituitary tumor: Strain No.	Type of tumor	TSH potency, USP U/g	Prolactin: Total dose, mg	Prolactin: Potency, IU/g
Mouse	77D	Thyrotrophic, dependent	200	200	<15
	101D	" "	100	"	"
	OG2	Adrenotrophic, autonomous	<.2	"	"
	OG3	Mammotrophic, "	"	"	"
	OG4	" "	.35	10*	10±
	"	Sample A, ♂—acetone dried	<.2	200	<15
	"	" B, ♀— " "	"	"	"
	"	" C, ♂—lyophilized	"	"	"
	"	" D, ♀— "	"	"	20
Rat	FR4	Mammotrophic, dependent	<.1	"	50
Bovine anterior pituitary gland powder			10-20	20	500

* Local cropsac assay.

TABLE II. Prolactin Potency of Crude Extracts of Mammotrophic Tumor Powders.

Tumor powder: Species	No.	Type	Amt extracted, g	Extract: Yield, dry wt, g	Prolactin: Total dose, mg	Prolactin: Potency, IU/g	Prolactin: Tumor powder, IU/g*	TSH: Potency, USP U/g	TSH: Tumor powder, USP U/g*
Mouse	OG4A,B,C	Autonomous	10.5	1.2	200	44	5	<.15	<.02
Rat	FR4	Dependent	1.3	.24	20	300	56	"	<.03

* Potency of original tumor powder calculated from potency of extracts.

Crude extracts were made of pooled mammotrophic mouse tumors (OG4, samples A, B, and C) which had given negative tests for prolactin and of rat tumors (FR4) in order to test the feasibility of concentrating the activity before assaying. Both extracts tested negatively for TSH and positively for prolactin (Table II). The autonomous mouse tumor OG4 contained perhaps as little as 5 I.U./g of prolactin, or about 10% that of the dependent tumor FR4 and 1% that of bovine pituitary powder. The extract of the dependent tumor FR4 had a potency of 300 U/g which indicated a potency of 56 U/g for the original tumor powder. This confirmed the prolactin potency of 50 U/g obtained by direct assay of FR4 powder.

TSH content of tissues of the mouse: Because the blood level of TSH is high and the tumors are fairly vascular, it was thought worthwhile to determine the TSH content of various tissues of the body (Table III). Over 98% of the TSH activity was found to be in the tumor and only about 1% in the blood stream. The small amount found in the body could be due to unidentified metastases and residual blood.

Discussion. From the data in Table III it can be calculated that a 5 g tumor weighs 2,500 times the pituitary of the mouse and contains at least 5,000 times as much TSH. Also, the serum concentration of TSH is over 2,000 that of normal human blood. Only negative tests have been obtained with normal mouse serum.

TABLE III. TSH Potency of Pituitary Gland, Blood Serum, Tumor and Body of Mice with TSH Producing Tumors.

Tissues measured	TSH potency—USP units: Wet wt basis	Dry wt basis, U/g	Total potency, U/mouse	
Tumor	40 U/g	200	200	(1)
Blood serum	1-2 U/ml	10	1-2	(2)
Body minus tumor	.1 U/g	.5	2	(3)
Pituitary	20 "	100	.04	(4)

(1) Basis of a 5 g tumor. (2) Basis of 1 ml serum. (3) Basis of 20 g body wt. (4) Basis of 2 mg pituitary.

The TSH concentration in human blood serum is about 0.5 U/liter, or about 2 units per man(8). A human pituitary contains only about 5 to 10 units of TSH so that the total amount of TSH in the blood stream is as much as 20 to 40% of that in the pituitary gland. Hence, it is surprising that in mice with TSH tumors the blood content is only 1% that of its source, *i.e.*, the tumor. In other words, the pituitary content/blood content ratio in normal man is 5/1, but the tumor content/blood content ratio in mice is 100/1. The supernormal hormone concentration in the tumor tissue and blood of mice with transplantable dependent thyrotrophic pituitary tumors is most unusual and these materials may, therefore, be unusually valuable in physiological and chemical studies. It may be noted that these pituitary tumors release TSH at a site distant from the hypothalamus.

Meyer and Clifton(9) have recently shown that primary pituitary tumors induced by stilbestrol pellets in rats have the same prolactin concentration as normal rat pituitaries. These results cannot be correlated with our present data because their potency values were only relative and not in terms of International Units.

Summary. The prolactin and thyrotrophin content of various transplantable pituitary tumors was studied. 1. Prolactin was found in stilbestrol induced and stilbestrol dependent rat pituitary tumor and also in an autonomous mammotrophic mouse tumor in concentrations only 10% or less that found in bovine pituitaries. These tumors contained little or no thyrotrophin. 2. Two autonomous adrenotrophic tumors contained no detectable prolactin or thyrotrophin. 3. Two dependent thyrotrophic tumors contained no detectable prolactin while the thyrotrophin concentration was ten times that of bovine pituitaries. A five gram thyrotrophic tumor contains 5,000 times more thyrotrophin than one mouse pituitary. Blood levels of thyrotrophin were 2,000 times normal.

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Received October 26, 1956. P.S.E.B.M., 1956, v93.

Mode of Action of Phenaglycodol, A New Neurosedative Agent.† (22807)

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Among a series of diols studied by Gibson, Mills and Swanson*, phenaglycodol (2-p-chlorophenyl-3-methyl-2, 3-butanediol) was outstanding because of the relatively long duration of its anticonvulsant action. While the compound was being tested for antiepileptic activity, the ward attendants reported that the children had become much easier to handle. Further clinical investigation has confirmed the impression that phenaglycodol can be classified as a tranquilizing agent of the muscle relaxant group. Since it is more potent and longer acting than some of the currently known drugs of this type further study seemed indicated.

Methods. Considerable attention has been given to careful observation of changes in behavior of unanesthetized mice, cats and monkeys given phenaglycodol. In addition, we studied the effect of the drug on selected multisynaptic reflex arcs in the cat. Flexion reflex was elicited at 10-second intervals by a 0.2 millisec. square wave 10-volt pulse to the posterior tibial nerve and recorded from the anterior tibial tendon with an isotonic lever in cats anesthetized with 50 mg/kg of chloralose. The knee jerk was obtained by tapping the patella tendon with a solenoid tapper. This monosynaptic reflex was modified by mid-brain stimulation(1,2). The biphasic pulses of an integrated square wave(3) of 1 millisec. duration at 20-50 cycles/sec. were led to a concentric bipolar electrode or to a monopolar electrode with the stereotaxic frame as an indifferent electrode. Points in the mesencephalic reticular formation were located by trial and error, which gave either facilitation or inhibition. Inhibition was induced by caudate stimulation. In the bulbar reticular formation, stimulation caused inhibition of the knee jerk and usually, apnea. In spinal cats, a comparison was made of the effect of phenaglycodol on predominantly monosynaptic inhibition of the knee jerk by ipsilateral sciatic stimulation, with the effect on a multisynaptic inhibition by contralateral stimulation of the nerve to the quadriceps muscle(1). Electrical activity of the brain was recorded in unrestrained cats from surface and deep electrodes chronically implanted. Some experiments were done on cats immobilized with suspension of dimethyltubocurarine butyrate in oil. The surface electrodes were 2-56 stainless steel machine screws that were placed in the skull to reach, but not penetrate, the dura. The deep electrodes were of 24-gauge stainless steel wire insulated to within 1 mm of the tip with Epoxylite Electrode Insulating Varnish. In chronic animals, Cannon miniature plugs or Winchester subminiature sockets were secured to the skull with dental acrylic resin. The surface electroencephalograms were picked up from bipolar connections or from one screw and an "indifferent" electrode over the frontal sinus. For the most part, the deep electrodes were recorded as a unipolar derivation with the indifferent electrode. The deep electrodes were placed in the septal region, the mesencephalic reticular formation, the ventro-posterior lateral and medial dorsal nuclei of the thalamus, the amygdala, and the caudate nucleus.

Results. Mice, after the injection of phenaglycodol in doses of 55 to 80 mg/kg intraperitoneally, show a reduction in spontaneous activity and sit quietly in the center of the observation area. Although muscle weakness can be demonstrated, the animals are usually not ataxic. With larger doses, there is a rapid loss of pinnal and righting reflex while the corneal reflex is still brisk. The onset of this action is smooth and there is little of the ataxic running about usually seen with phenobarbital.

Cats given doses of 20 mg/kg intraperitoneally also became quiet. It was often

† The trade-mark for Eli Lilly and Company for phenalglycodol is 'Ultran.'

* To be published.

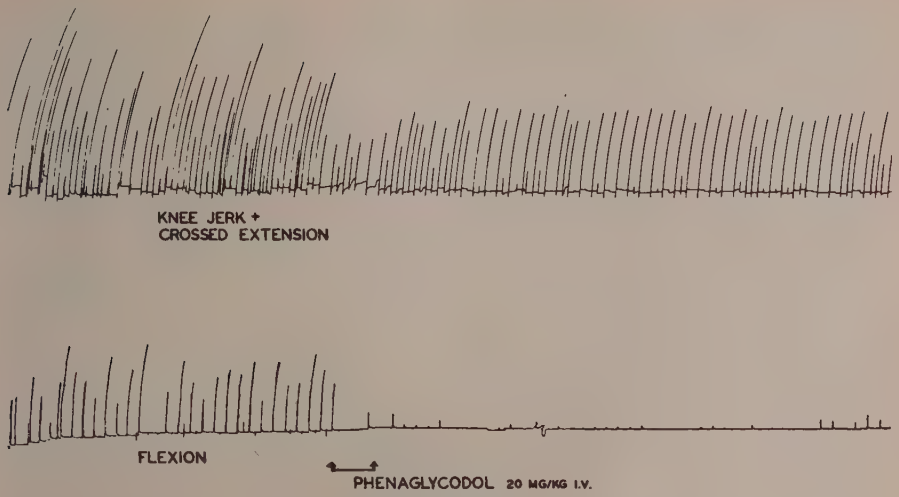

FIG. 1. Effect of phenaglycodol on spinal cord reflexes in cat. Upper trace is record of extension of the leg. Before drug, the leg extended in response to both tapping patella tendon and stimulation of contralateral posterior tibial nerve. After phenaglycodol the crossed extension disappeared while knee jerk became more consistent in amplitude.

noted that animals that were complaining about being confined in a cage seemed peaceful and content after phenaglycodol, in doses of 10 and 20 mg/kg. The animals given 40 mg/kg were ataxic and those on larger doses slept. No changes in respiration or in heart rate were noted.

Monkeys given phenaglycodol in doses of 50 to 100 mg/kg by stomach tube were less aggressive or less fearful. These animals often became playful and engrossed in their own activity. As with the other tranquilizing agents, there were a variety of patterns of behavior, depending not only on the dose of drug, but also on the character of the monkey, and on the observer and his previous experience with the colony. The larger dose caused some unsteadiness in movement. These doses were repeated daily for 6 months without appreciable change in intensity of effect. The animals usually recovered in 4 hours.

In cats anesthetized with chloralose, 20 mg/kg intravenously of phenaglycodol caused a small transient fall in blood pressure associated with some slowing of respiration. Heart rate and electrocardiogram were virtually unchanged. The pressor response to either carotid occlusion or epinephrine and the depressor response to either histamine or methacholine were unaltered. The contraction of the nictitating membrane after epinephrine or during preganglionic stimulation was not altered by phenaglycodol. Flexion reflex was usually reduced, but when the stimulus was supramaximal, there was not much change.

In experiments in which the posterior tibial nerve was stimulated, flexion reflex could be recorded ipsilaterally and crossed extension contralaterally. This stimulus was alternated with tapping of the contralateral patella tendon. Phenaglycodol in doses of 20 mg/kg intravenously caused little change in the knee jerk, but flexion and crossed extension were decreased (Fig. 1). These results were obtained in animals anesthetized with chloralose or dial-urethane solution. Further evidence of a selective depression of multisynaptic pathways in the cord was obtained from experiments in spinal cats. Phenaglycodol blocked the inhibition of the knee jerk caused by stimulation of the nerve to the contralateral quadriceps muscle but had little effect on the inhibition obtained by ipsilateral sciatic stimulation.

In experiments in which the knee jerk was

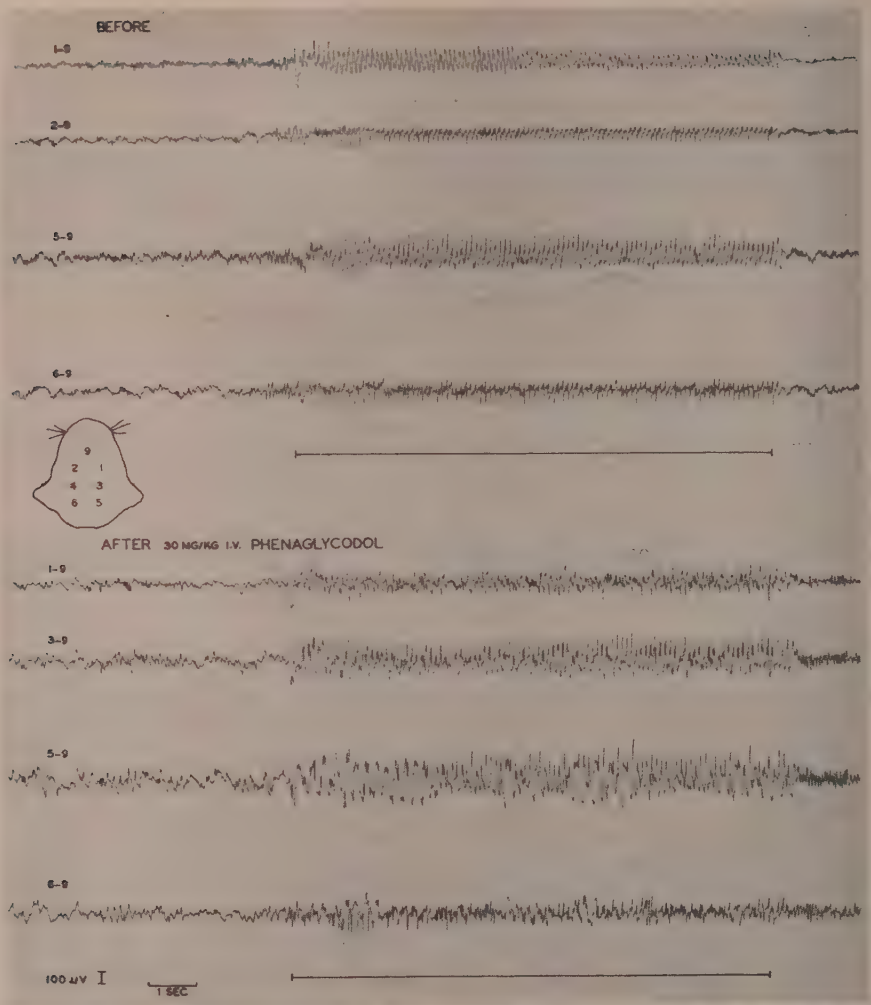

FIG. 2. Effect of phenaglycodol on EEG of cat showing change in form of recruiting response and of spontaneous record. Heavy line beneath records indicates duration of the 9 cycle/sec. stimulus.

inhibited or facilitated by supratentorial stimulation either from the mesencephalic reticular formation or from the caudate nucleus, doses of phenaglycodol of 10 to 20 mg/kg intravenously blocked the alteration of the knee jerk without changing the knee jerk itself. We did not detect a consistent difference in the effect of the drug on either facilitation or inhibition. When inhibition was obtained by stimulation of the bulbar reticular formation, however, phenaglycodol and mephenesin failed to block the change. It was characteristic of phenaglycodol that, when the drug caused a reduction in the activity of a polyneuronal arc, a simple increase in voltage of the stimulus would restore the response to or almost to the pre-drug level. The failure of phenaglycodol to block subtentorial inhibition may be related to the difference in the effect of a small increase in stimulus strength on the bulbar and mesencephalic reticular formation. In the former region a

small increase in voltage changes a barely discernible decrease in knee jerk to a complete block of the reflex. In contrast, small increase of voltage of mesencephalic stimulation caused less striking changes. This difference probably reflects the smaller number of interneurons involved in mediating the effect of bulbar stimulation.

In cats immobilized with dimethyl-tubocurarine butyrate in oil, low voltage fast activity was the usual pattern of the electroencephalogram. After phenaglycodol in doses of 10 to 20 mg/kg intravenously, this desynchronized record showed a greater degree of synchrony. The voltage increased from 30-50 μV to 100-150 μV, and the frequency slowed to 9-12 c/sec. In 3 cats of this type, 9 c/sec. stimulation in the nonspecific thalamic nuclei gave a typical recruiting response in the surface leads. The threshold for this response was not changed although the form was somewhat modified after phenaglycodol (Fig. 2).

In the unrestrained cats there was a greater variety in the resting EEG pattern, but the activation pattern was usually observed. After 20 mg/kg intraperitoneally of phenaglycodol the change to a higher voltage, slower frequency record was observed. We were unable to convince ourselves that any one of the surface or deep leads could be identified as the source or starting point for the synchrony. A somewhat similar pattern of action has been obtained with pentobarbital, 10 mg/kg, but the cat seemed somewhat more sleepy after this dose. Another difference was noted in that tapping or scratching of the cage would cause an EEG arousal response after phenaglycodol, but failed to do so after pentobarbital. From these observations it would appear that phenaglycodol lies somewhere between the muscle relaxants, which depress the recruiting response but leave EEG arousal unimpaired, and the barbiturates which depress arousal and do not depress and perhaps even enhance recruiting (4).

Discussion. It seems clear from the results of these experiments that phenaglycodol is a sedative that differs materially from the barbiturates in its mode of action. The pattern of action in mice, cats and monkeys demonstrates this distinction. More detailed studies on polysynaptic pathways indicate that phenaglycodol has properties in common with the interneuronal blocking agents such as mephenesin, at least at the level of the spinal cord. The changes in the electroencephalogram demonstrate an action on higher structures, but further work will be needed to localize this effect.

Summary. 1. Phenaglycodol, a compound originally selected for clinical trial on the basis of its anticonvulsant and effective tranquilizing actions, has been shown to have a quieting effect on mice, cats and monkeys. 2. Its effects on blood pressure, respiration and electrocardiogram are minimal. 3. In studies on selected reflex arcs it has been shown to be a selective depressant of polysynaptic pathways at the spinal and supraspinal level. 4. After doses of phenaglycodol causing sedation but not sleep in cats, the electroencephalogram shows a pattern of 9-12 c/sec. synchronous activity at both surface and deep electrodes.

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Received September 28, 1956. P.S.E.B.M., 1956, v93.

Fractionation of Plasma Non-Esterified Fatty Acids. (22808)

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Previous studies(1,2) have shown that non-esterified fatty acids in plasma (NEFA) fall sharply after administration of glucose or insulin, and rise after an injection of epinephrine. On the other hand, fat meals caused no significant rise of NEFA, even during the phase of hyperlipemia or in the subsequent clearing of plasma. The detailed composition of this fraction has remained undefined, except for indirect evidence that it is composed mainly of long chain fatty acids loosely coupled to albumin. Chromatographic analysis, used in the present study, shows that palmitic and oleic acids predominate in NEFA, as they do in plasma triglycerides and adipose tissue(3), and that fall in concentration of these acids accounts for the main part of the fall in concentration of NEFA after injection of insulin.

Methods. NEFA was extracted from plasma and titrated into 90% ethanol as previously described(2), then re-extracted into petrol ether (BP 50-60°C) and concentrated at 55°C under a stream of nitrogen to an acidity of 15 to 25 μeq./cc. After addition of a small amount of C^{14}-palmitic acid* to serve as a marker, the concentrate was fractionated on an octane-methyl cellosolve-water partition column(4), and alternate tubes of collected effluent were titrated for acidity or bromine uptake(5). Since palmitic and oleic acid overlap in this fractionation, a more accurate measure of their relative proportions is obtained by preliminary bromination of the extract at –20°C, thus moving the oleic acidity to a relatively empty place in the plasma spectrum. Both methods were used.

Results. Fig. 1 shows the fractionation of plasma NEFA obtained from a normal fasting subject. Fig. 2 and 3 show the changes in

TABLE I. Distribution of Fatty Acids in NEFA before and after Injection of Insulin.

Time (min.)	Plasma sample	NEFA (μEq/l)	Relative proportions (%) Stearic	Palmitic	Oleic	Not identified
–2	1	1068	6	34	54	6
0			(Insulin, 0.1 u/kg, inj. i.v.)			
45	2	527	8	36	54	2
90	3	468	10	29	42	19

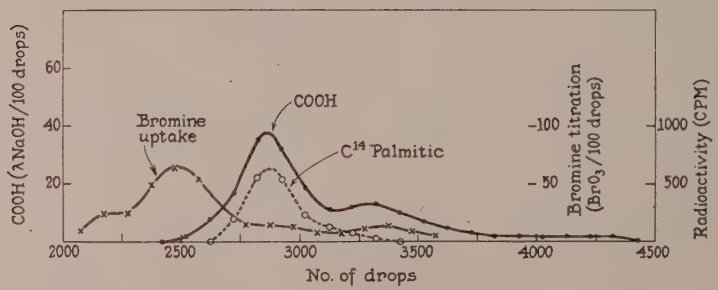

FIG. 1. Chromatographic fractionation of plasma extract. Titration of acidity (●) and uptake of bromine (×). The C^{14}-palmitic acid, added as a marker (○), contributed negligible acidity. The two partially separated peaks of acidity appear to represent palmitic and oleic acids.

* Obtained from Tracerlab, Inc., Boston.

pattern after intravenous injection of insulin. The total NEFA and proportions of major fatty acids in one experiment are given in the table.

Summary. Palmitic and oleic acids are the predominant non-esterified fatty acids of human plasma. Their concentrations fall proportionately after an injection of insulin.

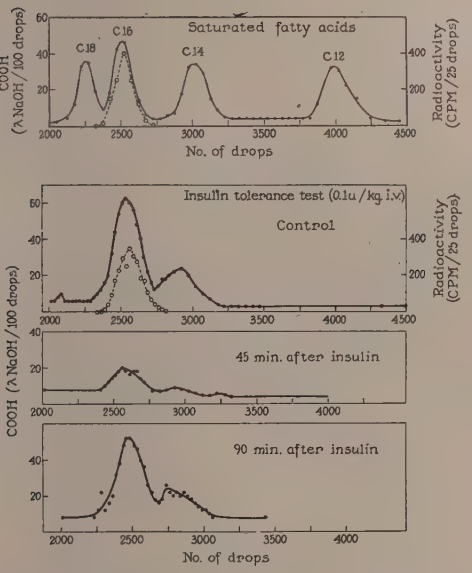

FIG. 2. Fractionation of plasma taken before and after an inj. of insulin. The upper curve shows a calibration of the column with 4 standard saturated fatty acids (solid curve) and a C^{14}-palmitic acid marker (dotted curve).

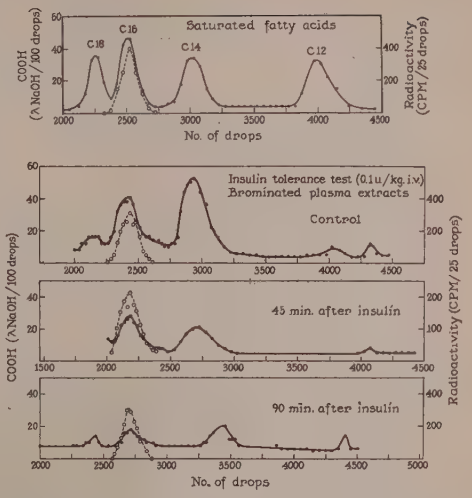

FIG. 3. Same kind of exp. as that shown in Fig. 2. The extracts were brominated before chromatography in order to increase the polarity of oleic acid and thus move the peak away from that of palmitic acid.

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Received September 28, 1956. P.S.E.B.M., 1956, v93.

Effect of Oral Hypoglycemic Drug (Carbutamide) on Glycogen Deposition By Isolated Rat Hemidiaphragm. (22809)

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Recently sulfonylurea derivatives have been introduced in the treatment of diabetes mellitus(1-4). Although these compounds are capable of lowering the blood sugar, their mechanism of action is unknown. Originally it had been postulated that this effect was mediated through destruction of the alpha cells of the pancreas(1-3), but subsequent work has failed to confirm this(5). Mirsky *et al.* have reported an inhibition of insulinase, an enzyme system in the liver capable of degrading insulin, and concluded that such inhibition of insulin destruction was the mode of action of the sulfonylurea compounds(6). Vaughan, using a smaller amount of Carbutamide (N-p-amino-benzene-sulfonyl-N′-n-butyl urea) was unable to detect any inhibition of insulinase(7). However, in other experiments she found that Orinase (N-toluene-sulfonyl-N′-n-butyl urea) significantly reduced the effect of both epinephrine and glucagon on the liberation of free glucose from liver slices(7). The physiological significance of these findings is open to some question in view of the observation that the hyperglycemic response to epinephrine and glucagon is not altered in diabetic patients successfully treated with the sulfonylurea compounds(8).

The present studies were done to ascertain whether Carbutamide* had any insulin-like effect on the metabolism of the isolated rat hemidiaphragm or whether it would augment the effect of a known amount of insulin.

Methods. Insulin *in vitro* is known to increase the glycogen deposition by the isolated rat hemidiaphragm as compared to its control hemidiaphragm not so exposed to insulin, but otherwise handled identically(9). This increment of glycogen deposition as a result of insulin, expressed in micromoles of glucose equivalents/g of tissue, is referred to as the insulin effect. The method of determining the insulin effect was the same as reported previously(10), except for the following modifications. In the experiments designed to determine the effect of Carbutamide alone on the glycogen deposition of the diaphragm no insulin was used. Instead, one hemidiaphragm was exposed to 2 ml of 0.04 M phosphate buffer (pH 6.8) containing 2 mg of Carbutamide for 1 minute while the control hemidiaphragm was exposed to buffer alone, prior to incubation of both for 90 minutes in 0.4% glucose dissolved in buffer. At the end of the incubation, the glycogen content of each hemidiaphragm was determined. In other experiments, one hemidiaphragm was shaken with 0.2 unit of insulin in 2 cc of buffer for 1 minute while the control diaphragm was exposed to only buffer. Subsequently each hemidiaphragm was incubated for 90 minutes in 2 ml of buffer containing 2 mg of Carbutamide and 0.4% glucose. Two other series of experiments were done; in one set one-tenth (0.2 mg) the amount of Carbutamide was used, while in the other series 2 mg of sulfadiazine were substituted for an equal amount of Carbutamide. Since the usual therapeutic blood level of Carbutamide in humans is about 10 to 15 mg %, the lower concentration used represented a physiologic amount while the higher one was in the range of that used by Mirsky *et al.*(6) and Vaughan(7).

Results. Table I summarizes the results. Carbutamide by itself did not exert any insulin-like action on the glycogen deposition of the isolated rat hemidiaphragm. The effect of 0.2 unit of insulin was an increase of 6.29 micromoles of glucose equivalent per gram of tissue. When one hemidiaphragm was exposed to 0.2 unit of insulin and then incubated in glucose and buffer containing 2 mg of Carbutamide, the insulin effect was reduced to 2.90 micromoles per gram of tissue. The

* Carbutamide used was generously supplied by Eli Lilly and Co., Indianapolis, Ind.

TABLE I. Effect of Insulin and Carbutamide on Glycogen Deposition of Rat Hemidiaphragm.

Material used with treated diaphragm	Treated diaphragm	Control diaphragm	Insulin effect
	μmoles (glucose equivalent)/g tissue		
Carbutamide, 2 mg	16.63 ± .74*	16.29 ± .90*	.34 ± .76* (6)§
Insulin, .2 unit	23.54 ± .97	17.24 ± .82	6.29 ± .59 (25)
Idem + Carbutamide, 2 mg	18.50 ± .95	15.60 ± 1.05†	2.90 ± .70 (18)
" + Carbutamide, .2 "	15.65 ± 1.71	10.74 ± 1.26†	4.91 ± .74 (9)
" + sulfadiazine, 2 "	17.37 ± 1.11	15.44 ± 1.69‡	1.93 ± 1.35 (6)

* Mean ± S.E.
† Control hemidiaphragm also treated with Carbutamide.
‡ " " " " " sulfadiazine.
§ No. of determinations in parentheses.

control hemidiaphragm was also incubated in glucose and buffer with 2 mg of Carbutamide, but it was not previously exposed to insulin. This represents a significant reduction of the insulin effect by Carbutamide ($P = < .01$). When the same experiment was repeated using a lower concentration of Carbutamide (0.2 mg), the insulin effect was 4.91 micromoles per gram of tissue, not significantly different from the insulin effect in the absence of Carbutamide. It should be noted that at this lower concentration of Carbutamide there was no enhancement of the insulin effect. If Carbutamide acts by inhibiting insulinase, and since insulinase has been reported in muscle (11), one might expect an augmentation of the insulin effect by these sulfonylurea compounds. Even though the rat hemidiaphragm is capable of a greater response to larger amounts of insulin,† the system as used in these experiments might not be sensitive enough to detect a small increment in the insulin effect. Nevertheless, when larger amounts of Carbutamide were used there was an unexpected and highly significant reduction of the insulin effect. Although these experiments do not indicate the mechanism of action of Carbutamide, they do tend to eliminate a direct insulin-like effect.

In an effort to determine the specificity of the inhibition of the insulin effect observed with the larger dose of Carbutamide, similar experiments were done using an equivalent amount of sulfadiazine. With this sulfonamide the insulin effect was also significantly reduced to 1.93 μmole/g of tissue ($P = < .01$). It thus seems that the inhibition of the insulin effect is not limited to the hypoglycemic sulfonylurea derivatives but is also shared by other sulfonamide drugs. The mechanism of this inhibition is not clear at this time. In the higher concentrations, these sulfonamide derivatives may act as non-specific protein poisons affecting several different tissue enzymes. On this basis it might also be possible to explain the inhibition of insulinase reported by Mirsky *et al.*(6) and the reduced epinephrine and glucagon response observed by Vaughan(7). The amounts of the sulfonylurea derivative used by Mirsky were 5 to 25 mg while Vaughan used 6.75 mg/100 mg of liver slices. In the present studies 2 mg were capable of inhibiting the action of insulin on the rat hemidiaphragm while one-tenth this amount had no such effect.

Summary. Carbutamide, a sulfonylurea compound capable of lowering blood sugar, did not have an insulin-like action in increasing glycogen deposition of isolated rat hemidiaphragm. Furthermore, this drug did not produce any enhancement of the insulin effect. In fact, when higher concentrations were used there was a significant inhibition of the insulin effect. This inhibition is somewhat non-specific since it was also observed when sulfadiazine was substituted for Carbutamide. It is suggested that this inhibition, the inhibition of insulinase, and the *in vitro* reduction of the epinephrine and glucagon response which have been attributed to the sulfonylurea compounds might all be due to a non-specific protein poisoning action of

† When 2 units of insulin were used instead of 0.2 unit, the insulin effect was 10.63 ± 0.46.

these drugs in higher concentrations.

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Received October 1, 1956. P.S.E.B.M., 1956, v93.

Epinephrine Sensitivity of Blood Vessel Strips from Salt-Fed and Castrated Rats.* (22810)

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The observation that high sodium chloride intake increased blood pressure in rats has been made by several investigators, including Tobian, Fregly, Meneely and associates, Sapirstein, and others(1-4). The salt diet apparently induced hypertension by increasing cardiovascular reactivity, since Raab(5) found that salt and DCA potentiated the pressor effects of epinephrine in rats. That blood vessels were involved was also suggested by findings of Tobian and Binion(6) that the aorta of rats on high salt diet contained more sodium than those on low salt.

In vitro studies of reactivity of blood vessels have been made by Franklin(7) and Furchgott and Bhadrakom(8), who showed that vessel strips constricted a measurable degree, when immersed in solutions of dilute epinephrine. Since high salt diet apparently increased sensitivity of the entire cardiovascular system to epinephrine, it appeared likely that vasoconstrictor functions of blood vessels might be implicated in the response. The object of this study was to determine if blood vessels from salt-fed rats differed from those of control animals with regard to epinephrine sensitivity.

Materials and methods. In the first experiments with 50 albino rats of Sprague-Dawley strain, about same age and size (80-100 g, and 60 days old), were used. The rats were separated at random into 2 groups, one fed a normal laboratory rat diet and tap water, while the other was given the same diet except that 2% sodium chloride by dry weight had been added, and drinking water changed to 0.9% saline. At the end of 10 weeks, control and salt-fed rats were removed in pairs at daily intervals and killed by blow on the head. The aortae were removed immediately from the level of the aortic arch to the origin of renal arteries. A segment 42 mm long was cut out and split longitudinally as shown in Fig. 1A. Incomplete transverse cuts were then made alternately from each side of flattened strip as shown in Fig. 1B. Control and salt-fed preparations were suspended in aerated Evans' Ringer's solution in 37° bath. Strips were attached to long heart levers, counterbalanced so that tension supplied by weight of levers was approximately 4 g. This weight acted to elongate the cut strips in such a way that circular and spiral

* This investigation was supported in part by grant from Amer. Heart Assn.

† Public Health Service Research Fellow.

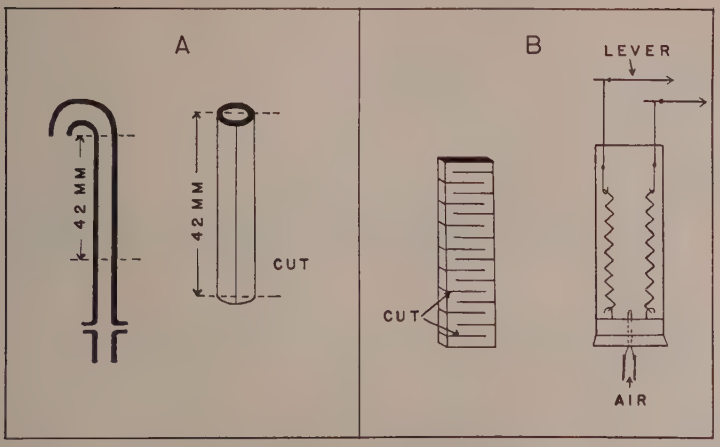

FIG. 1. Method of preparing aortic strips. A. Section cut from rat aorta and split lengthwise. B. Left, incomplete transverse cuts made across flattened strip of aorta. Right, aortic strips attached to levers and suspended in Evans' Ringer's solution.

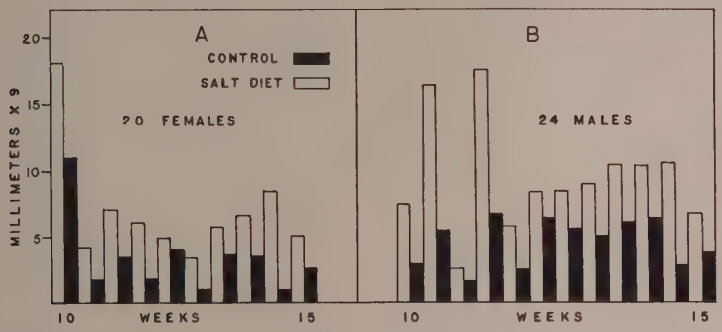

FIG. 2. Contractions produced by epinephrine in aortic strips. A. Response to epinephrine of aortic strips from control and salt-fed female rats. B. Same procedure using aortic strips from control and salt-fed male rats. Ordinate, amount of shortening of strip multiplied by magnification factor of lever system. Abscissa, number of weeks rats were kept on salt diet.

muscle layers of the aorta were oriented in a vertical direction (Fig. 1B). In preliminary experiments it was found that smooth muscle was usually contracted by the cutting procedures, and that best results were secured if the strip was allowed to relax for about 1 hour in aerated Ringer's solution previous to the testing procedures. Experimental and control strips were placed together in Ringer's solution to which l-epinephrine‡ was added, to make a concentration of 0.2 μg/ml. Usually 2 or 3 trials could be made for each pair of vessels provided relaxation of the muscle was induced by replacing the epinephrine solution with fresh Ringer's between tests. In a second series a similar group of 50 rats was separated as to sex, and half of the males and females castrated under ether anesthesia. The animals were allowed to recover for 3 weeks before being placed on the salt diet described above. After 10 weeks or longer the aortae of salt-fed castrated animals were compared with those of control diet castrates for sensitivity to epinephrine. As a further check on results, a group of intact rats from the same source was given either

‡ Supplied as Suprarenin, courtesy of Winthrop-Stearns, Inc.

FIG. 3. Effects of castration on epinephrine contraction of aortic strips. A. Comparison of contractile response of aortic strips from control and salt-fed female rats. B. Procedures as in A, except all rats were ovariectomized previous to being placed on salt or control diets. C. Procedures as in A, except all animals were intact male rats. D. Procedures as in A, except all rats were castrated previous to being placed on salt or control diets. Ordinate and abscissa as in Fig. 2.

salt or control diets and their aortae tested as described above.

Results. Effects of salt diet on aortic epinephrine sensitivity for intact rats are shown in Fig. 2. These data indicate that responsiveness to epinephrine of aortic strips from salt-fed rats of both sexes was about double that of control strips. Statistical treatment of the data by Fisher's "t" method (9) gave a P value of 0.001, indicating a highly significant difference in sensitivity between salt-fed and control rats of both sexes. The results shown in Fig. 2 also disclosed a sex difference in the response of the aortae from salt-fed and control groups, the male aortic strips being more responsive to the same epinephrine solution than female strips. Statistical treatment gave $P = 0.001$, indicating a significant difference between the two groups.

By comparing Fig. 3A and B it can be seen that castrated females on control diet yielded aortae that were slightly but not significantly more sensitive to epinephrine than did normal females ($P = 0.2$). However, when castrated females on salt diet are compared with normal salt-fed females, (Figs. 3A and B) it is clearly evident that ovariectomy greatly increased the sensitivity of aortae to the salt diet. Statistical treatment of data gave a P value of 0.01, indicating that the difference was not likely due to chance.

In contrast to results found in operated females, the castration of males did not cause any similar increase in sensitivity of the aortae to epinephrine. When the salt diet was imposed on castrated males there was also no appreciable increase in reactivity of the aortae over those of normal males on the salt diet, as can be seen by comparing Fig. 3C and 3D. The normal rats shown in Fig. 3A and 3C confirm the finding that high salt diet increased epinephrine sensitivity of the aorta in both sexes.

Discussion. The finding that a high salt diet resulted in deposition of increased sodium in tissues, and that this was associated with development of hypertension, has been established by several investigators cited previously. From our observations on isolated blood vessel strips, it is apparent that one result of sodium deposition is an increased sensitivity of circular smooth muscle to the stimulating effects of epinephrine.

Sex differences found in the aortic response to epinephrine are in accord with the findings of Stamler(10) that salt-induced hypertension could be produced readily in male dogs and cockerels, while at most only a slight response could be produced in female dogs and laying hens. Estrogens were reported by Stamler to have anti-hypertensive effects, in that this hormone lowered blood pressure, or prevented the expected rise when DCA plus salt diets were imposed on cockerels.

Removal of ovaries and a high salt diet in our rats caused increases in aortic epinephrine response to the extent that castrated females

developed about the same aortic sensitivity as males. In comparison to females, castration did not have much effect on males, since little difference was noted when the aortae of operated animals were compared to normal ones. Likewise, the salt diet did not increase aortic responsiveness to epinephrine in castrated males beyond that found in normal males. These findings indicate that the presence of female sex hormones is required to maintain a low reactivity to epinephrine in the aorta. Removal of the male hormone, on the other hand, did not change the reactivity of the aorta, nor sensitize the animal to the salt diet. The mechanism whereby the salt diet increases the reactivity of vessel strips to epinephrine may be explainable by the theory of Raab(5) that increased intracellular sodium in smooth muscle of blood vessels potentiates the constrictor effects of epinephrine, possibly by raising the membrane electrical potentials of muscle cells upon which catecholamines act.

Summary. Strips were prepared from aortae of rats and their reactivity to epinephrine tested by means of a lever system that recorded shortening of the smooth muscle. A 2% salt diet increased the sensitivity of the vessel to l-epinephrine in both sexes. Male aortae in all instances had greater sensitivity to epinephrine than those of females under the same conditions. Castration markedly increased the sensitivity to epinephrine in females, especially when the castrates were placed on the salt diet. In contrast, male aortae were not particularly changed by castration.

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Received October 1, 1956. P.S.E.B.M., 1956, v93.

Insulin Secretion Following Carbutamide Injections in Normal Dogs.* (22811)

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The hypoglycemic action of orally active sulfonylureas may be due to one or more of the following reasons: a) destruction of the pancreatic A cells with decreased glucagon production(1), b) inhibition of one or more of the enzyme systems involved in liver glycogenolysis(2-6), c) decreased intestinal absorption of glucose(7), d) enhancement of insulin action(8), e) inhibition of the enzymes responsible for insulin degradation (9), and f) stimulation of insulin secretion (10-12). The possibility that these drugs may act through pituitary, adrenals, thyroid, parathyroid, testicles or the central nervous system appears unlikely(13) while the possibility that they may stimulate peripheral glucose utilization is uncertain(14,15). Purpose of this work was to investigate the hypothesis that carbutamide stimulates release of insulin into pancreatic blood.

Materials and methods. The problem was investigated by means of 7 pancreatic-femoral and 5 mesenteric-femoral cross-circulation ex-

* Aided by grant from N. I. of Arthritis and Metabolic Diseases (No. A-522), N.I.H., Public Health Service.

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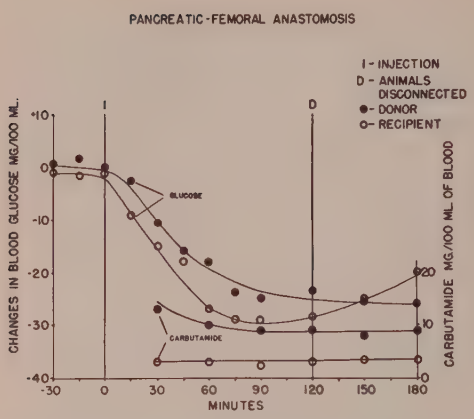

FIG. 1. Effect of carbutamide on blood glucose of dogs. Avg of 5 pancreatic-femoral cross-circulation experiments. ●, donor *dogs D*; ○, recipient *dogs R*. I = Inj. of carbutamide into *dogs D*; D = Dogs disconnected.

periments, using normal mongrel dogs of both sexes. Animals fasted for about 24 hours preceding the experiments were anesthetized with sodium pentobarbital (35 mg/kg, intraperitoneally) and heparinized.‡ The experiments were performed as described previously (16). Carbutamide was injected intravenously (50 mg/ml, 1 ml/kg, pH 8).§ Blood glucose was determined according to the method of Nelson(17), blood carbutamide according to the method of Bratton and Marshall(18). At the end of the experiment the animals were killed with excess anesthetic and portions of head, body and tail of pancreas were fixed in Bouin's solution for subsequent histological study, according to the method of Gomori(19).

Results. Results are presented in curves showing average changes in blood glucose concentration. Fig. 1 represents 5 pancreatic-femoral cross-circulation experiments in which donor *dog D* received an injection of carbutamide. During the control period preceding injection, there were no significant changes in blood sugar of either dog. Following carbutamide injection the blood sugar of dog D started to decline rapidly, reaching a minimum in about 1 hour and remaining low for the duration of the experiment. Maximum reduction varied between 25 and 60% in individual dogs D and was accompanied by similar hypoglycemia (25-68%)‖ in recipient *dogs R*. After interruption of the anastomosis, while the blood sugar of dog D remained low, that of dog R began to rise slowly toward preinjection levels. Fig. 2 represents 3 mesenteric-femoral cross-circulation experiments in which dog D received an injection of carbutamide. No significant changes in blood sugar of either dog D or Dog R were noted during the preinjection control period. Following the injection, blood glucose of dog D fell rapidly, while dog R showed a mild and not significant hyperglycemia. It should be pointed out that the maximum hypoglycemic effect of carbutamide did not occur at the same time in all animals and, therefore, the average curves are flatter than those representing individual experiments. Concentration of carbutamide in blood after initial mixing, reached values of about 8 mg % in dog D and about 2 mg % in dog R and remained essentially unchanged for the duration of the experiment. In 2 additional pancreatic-femoral and 2 additional mesenteric-femoral cross-circulation experiments, not included in the average curves, carbutamide did not cause hypoglycemia either in dog D or dog R, al-

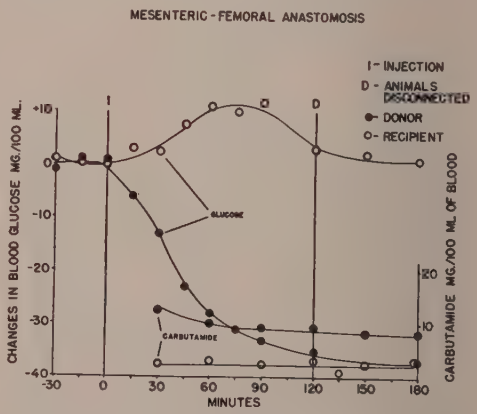

FIG. 2. Effect of carbutamide on blood glucose of dogs. Avg of 3 mesenteric-femoral cross-circulation experiments. Symbols as in Fig. 1.

‡ Heparin was gift of Dr. Stanley Hier of Wilson Laboratories.

§ Carbutamide was gift of Dr. W. R. Kirtley of Lilly Research Laboratories.

‖ $P < 0.01$.

though the same dose had been used in all experiments and the same blood concentrations had been obtained. No significant changes were noted in the general appearance, in granules and other staining characteristics of A and B cells of the islets of Langerhans.

Discussion. The results suggest that carbutamide hypoglycemia in dog D is accompanied by a release of insulin into the blood of the pancreatic vein and that this insulin is carried through the pancreatic-femoral anastomosis, causing hypoglycemia in dog R. Small amounts of carbutamide are carried through the anastomosis also, but carbutamide concentration in blood of dog R appears to be too low to cause significant changes in blood sugar. This is shown by the fact that when dog R receives mesenteric instead of pancreatic blood from dog D, no hypoglycemia occurs, even though concentration of carbutamide in blood of dog R is approximately the same in both types of experiment. Failure of blood sugar of dog R to decrease in those experiments in which dog D did not respond to carbutamide, the slow return toward normal of blood sugar in dog R after separation from its extra insulin supply and the sustained hypoglycemia in dog D under the lasting effect of the injected drug, lend further support to the stated hypothesis. These results are in agreement with those obtained by Loubatieres using p-amino-benzene-sulfamido-isopropyl thiodiazole(10) and with the fact that carbutamide has no hypoglycemic effect in the absence of pancreatic tissue(10) and in those diabetic patients most likely to have little or no insulin in their pancreas(20). The available data do not permit the differentiation between the release of preformed insulin and true insulin secretion, although the fact that the B cells did not appear degranulated suggests active secretion. This is suggested also by the observation that carbutamide stimulates the growth of islet tissue in normal(12) and alloxan-diabetic animals(10).

Summary and conclusions. The mechanism of the hypoglycemic action of carbutamide in normal dogs was studied by means of pancreatic-femoral and mesenteric-femoral cross-circulation experiments. The results of these acute experiments suggest that: 1) carbutamide hypoglycemia is accompanied by secretion of insulin; 2) carbutamide does not cause degranulation or other significant changes in appearance of pancreatic A and B cells; 3) clearance of carbutamide from blood is relatively slow, as reported by others(21); 4) some dogs are refractory to the hypoglycemic action of carbutamide in doses of 50 mg/kg I.V. The results are consistent with the hypothesis that insulin secretion is one of the results of carbutamide action. The continued administration of carbutamide in animals and men with reduced pancreatic reserve may stimulate the islets of Langerhans to regeneration or to exhaustion. Although some evidence suggests that islet growth does occur (10,12), the second alternative has not been ruled out and should be investigated before the sulfonylureas can be recommended for the prolonged treatment of diabetes mellitus.

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Received October 2, 1956. P.S.E.B.M., 1956, v93.

Plasma Phospholipide Synthesis in the Eviscerated Rabbit.* (22812)

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Fishler *et al.*(1) studied the synthesis of plasma phospholipides in fasting hepatectomized dogs with P^{32} and Goldman *et al.*(2) repeated this study with C^{14}-labelled palmitic acid. From this work it was concluded that the liver is the major source of plasma phospholipides in the fasting dog. Plasma phospholipides of rats in the post-absorptive state were also found to originate in the liver(3). On the other hand, Ranney *et al.*(4) demonstrated that, in the chicken, extrahepatic tissues contribute phospholipides to the plasma.

The following experiments were conducted to determine the site(s) of plasma phospholipide synthesis in the rabbit.

Methods. Normal white rabbits maintained on a diet of 100 g Purina rabbit chow per day, and fasted 24 hours prior to the experimental period, were sedated with intraperitoneally administered urethane (750 mg/kg) and anesthetized 30 minutes later by intravenously administered sodium pentobarbital (15 mg/kg). The gastrointestinal tract and spleen, and in some instances the kidneys, were removed through a median abdominal incision. The liver was left *in situ* isolated from the hepatic artery and portal vein. Before closing the incision, bandages wet with warm saline were placed in the abdominal cavity. Eviscerations were generally completed within 10 to 20 minutes, following which the rabbits were kept warm by application of external heat. Immediately after evisceration, rabbits received an intravenous dose of 100 mg glucose (10% in saline), followed by 100 mg/kg at 30 minute intervals through the remainder of the experiment(5). Control animals were sham operated, some receiving glucose, others not. Rabbits were injected intravenously with 0.5 to 1.0 mc of labelled phosphate and sacrificed four hours later by intravenous administration of sodium pentobarbital. Two series of experiments were performed. In series I plasma and tissues were extracted with alcohol-ether, petroleum ether and analyzed as previously described

TABLE I. Tissue Phospholipide Concentrations.

Tissue	Sham operated (mg P/g)		Eviscerated (mg P/g)	
Series I*				
Liver	1.36‖	± .02§	1.11‖	± .02
Kidney	1.11	± .03	1.12	± .01
Muscle	.283	± .010	.272	± .009
Lung	1.13	± .01	1.11	± .05
Plasma (4 hr)	.0359	± .0065	.0273	± .0050
Series II†				
Liver	1.20‖	± .06	.995‖	± .012
Kidney	1.02	± .07	1.03	± .11
Muscle	.310	± .022	.311	± .24
Lung	.990	± .008	.944	± .033
Plasma‡ (0 hr)	.0416	± .0052	.0494	± .0156
(4 hr)	.0481‖	± .0076	.0332‖	± .0089

* 5 sham-operated, 6 eviscerated rabbits.
† 4 " , 4 " " .
‡ 9 " , 9 " " .
§ Stand. error $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.
‖ Diff. significant at 5% level.

* Supported by grant H-1238 from National Heart Institute, N.I.H., U.S.P.H.S.

TABLE II. Specific Activity* of Tissue Phospholipides.

Tissue	Sham operated	Eviscerated
Series I†		
Liver	14.9‖ ± 2.1¶	.104‖ ± .02¶
Kidney	33.2‖ ± 3.7	58.1‖ ± 2.8
Muscle	.480 ± .063	.746 ± .121
Lung	8.07 ± 1.00	13.8 ± 1.0
Plasma	3.21‖ ± .65	1.22‖ ± .28
Series II‡		
Liver	12.9‖ ± 1.6	.120‖ ± .049
Kidney	29.8 ± 2.2	44.6 ± 5.7
Muscle	.707 ± .159	1.34 ± .28
Lung	9.97 ± 1.16	11.3 ± 2.0
Plasma§	3.36‖ ± .36	1.29‖ ± .26

* Specific activity $= \frac{\%\text{ injected } P^{32}}{\text{g lipide phosphorus}}$.

† 5 sham-operated, 6 eviscerated rabbits.
‡ 4 " , 4 " "
§ 9 " , 9 " "
‖ Diff. significant at 5% level.
¶ Stand. error.

(6,7). In series II, experimental procedures were modified as follows: a) zero time (t = 0) blood samples were taken for phospholipide analysis; b) plasma acid soluble specific activities were determined on trichloroacetic acid supernatants 2 and 4 hours after administration of P^{32}; c) plasma and tissues were extracted by the chloroform-methanol procedure of Folch *et al.*(8). Tables I and II as well as further unpublished observations indicate that the two extraction methods gave comparable results for specific activities even where the concentrations differed somewhat.

Results. No significant differences in concentration of lipide phosphorus of kidney, muscle, and lung were observed between normal and eviscerated animals (Table I). Only liver showed a decrease of 17-18% in the eviscerates. Although there was no difference in plasma lipide phosphorus concentration between sham operated and eviscerated animals of series I, inspection of series II would appear to show a decrease in the eviscerates. However, the more critical comparison of zero time and 4 hour plasma phospholipide levels in each eviscerated rabbit failed to show a significant drop in concentration.

Table II summarizes the specific activity data. In control rabbits, the specific activity of liver, kidney and lung exceeded the specific activity of plasma phospholipides. Muscle, however, showed a specific activity less than that of plasma excluding this tissue as a likely source of plasma phospholipides. In eviscerated rabbits liver specific activities were 7 to 9% of the control values, evidence that liver circulation was almost completely absent. The mean plasma phospholipide specific activities in series I and II were 8-12 times as great as those observed in liver. It is apparent that labelled phospholipides from sources other than liver found their way into plasma. Since the specific activities of kidney and lung phospholipides exceeded those of plasma phospholipides, these tissues could contribute phospholipides to the plasma pool.

In an attempt to localize further the possible sites of plasma phospholipide synthesis 9 rabbits were eviscerated: in 5 animals the kidneys were left in (eviscerated controls) and in 4 they were removed (eviscerated nephrectomized). Specific activities of plasma and liver phospholipides in eviscerated controls were 1.07 ± 0.11 and 0.071 ± 0.028, respectively, which agree with the animals of Table II. In the nephrectomized animals, however, the plasma phospholipide specific activity had decreased to 0.462 ± 0.016. It is thus evident that plasma phospholipide specific activity is reduced to ⅓ of normal upon removal of liver, intestine and spleen and that by removal of kidneys it is further reduced to about 1/10th of normal. That the small amount of labelled phospholipide appearing in plasma of eviscerated nephrectomized animals is not derived from liver is shown by the fact that the specific activity of plasma phospholipide of individual animals was 10-100 times that of liver.

In series I, kidney phospholipides showed a significantly higher specific activity in eviscerates than in controls. In order to explain this difference and the similar tendency towards higher specific activity in muscle and lung, ratios of tissue phospholipide specific activity to 4 hour plasma acid soluble phosphate specific activity were determined in series II. These ratios represent an index of the rate of synthesis of phospholipide from their phosphate precursors. No evidence of altered tissue phospholipide synthesis was

found in eviscerated rabbits. It appears, therefore, that the tendency towards higher specific activity in eviscerated groups is related to decrease in total phosphate pool and a subsequent increase in specific activity of phospholipide precursors.

Summary. In the rabbit, plasma phospholipide synthesis continued at a reduced rate in absence of liver, intestine and spleen. Removal of kidney further reduced plasma phospholipide synthesis. The rate of synthesis of kidney, lung or muscle phospholipides from phosphate precursors does not appear to be altered by evisceration.

The authors gratefully acknowledge the technical assistance of Mrs. Ruth McGavic, Ann Coulter, and Miss Pat Farrell.

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Received October 5, 1956. P.S.E.B.M., 1956, v93.

Metabolic Conversion of Primidone (Mysoline) to Phenobarbital.* (22813)

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5-Ethyldihydro - 5 - phenyl - 4,6-(1*H*,5*H*)-pyrimidinedione (primidone, Mysoline), a drug used in the treatment of epilepsy, differs structurally from phenobarbital only in that the former has a methylene group in the 2-position and the latter a carbonyl group. The only metabolic reaction that primidone has heretofore been reported to undergo is conversion to 2-ethyl-2-phenyl malonamide(1). Another reaction that suggested itself as a possibility is oxidation of the methylene group in primidone to yield phenobarbital as a product. The present investigation has shown that this reaction does indeed occur, and this report concerns a study of this metabolic conversion and its significance in therapeutics.

Methods. Administration of primidone. Crystalline primidone supplied through the kindness of Dr. A. Stanley Cook, Ayerst Laboratories, N. Y. City, was used in experiments on dogs. It was administered in gelatin capsules by mouth. The patients received commercial tablets. *Lack of contamination of primidone with phenobarbital.* A sample of crystalline primidone was partitioned between ether and water in a countercurrent design calculated to effect 99% separation of primidone and phenobarbital. Spectrophotometric examination of the fractions that would have contained phenobarbital demonstrated that the sample of primidone could have contained no more than 0.01% of phenobarbital. Information obtained from the manufacturer indicates that the method of synthesis excludes the possibility of any initial contamination with phenobarbital. *Spectrophotometric measurements* were made with a Beckman DU ultraviolet spectrophotometer. *Isolation of phenobarbital and p-hydroxyphenobarbital from urine.* The procedure described by Butler(2) was used. All samples of urine were subjected to acid hydrolysis. *Determination of phenobarbital in plasma.* For dog plasma the method of Butler *et al.*(3) was used. Unchanged primidone does not interfere to a significant extent with this method. A con-

* This investigation was supported in part by a research grant (B-384) from National Institute of Neurological Diseases and Blindness, of N.I.H., P.H.S.

† Public Health Service Research Fellow of N. I. Neurological Diseases and Blindness.

centration of 500 mg/l of primidone is measured as an apparent phenobarbital concentration of 2 mg/l. Plasma from patients receiving primidone and diphenylhydantoin was subjected to a countercurrent distribution procedure for the purposes of separating phenobarbital from diphenylhydantoin and of furnishing evidence of the identity of the phenobarbital in plasma. Samples of 10 to 15 ml of plasma were extracted with ether, the ether evaporated, and residues put through a 30 cell extraction train of the type described by Craig *et al.*(4). The light solvent was a mixture of 30% (v/v) ethyl ether and 70% 2,2,4-trimethylpentane and the heavy solvent a buffer consisting of 0.75 mole K_2HPO_4 + 0.25 mole KH_2PO_4 per 1 (pH 7.2). Each effluent fraction of light solvent was extracted with 4 ml of a buffer of pH 11 (0.10 mole $NaHCO_3$ + 0.09 mole NaOH per l). Concentration of phenobarbital was calculated from the difference between absorbancy of the buffer extract at 240 mμ and that at 260 mμ. With this system of solvents phenobarbital appears in maximal concentration in the nineteenth effluent fraction of light solvent. From the amount of phenobarbital in the peak fraction the concentration in original sample of plasma was calculated.

Results. Phenobarbital and p-hydroxyphenobarbital in urine. Primidone was administered to a dog in a daily dose of 200 mg/kg for 6 days. Urine was collected for 7 days beginning the day after first dose. From this urine there were isolated in crystalline form 77 mg of phenobarbital and 16 mg of *p*-hydroxyphenobarbital. These products were identified by melting points and mixed melting points. From a 21 hour specimen of urine from a patient receiving a daily dose of 1 g of primidone there was isolated in crystalline form 2.5 mg of *p*-hydroxyphenobarbital, which was identified by its absorption spectra in media of different values of pH. Phenobarbital was not isolated in crystalline form, but in the fraction in which it would be expected there was a substance with absorption spectra characteristic of phenobarbital. The amount of phenobarbital as calculated from absorption measurements was 0.9 mg.

Phenobarbital in plasma. By spectrophotometric procedures the presence of phenobarbital has been demonstrated and its concentration measured in samples of plasma from 2 dogs and 2 patients receiving primidone. The dogs were given, respectively, 200 and 250 mg/kg of primidone daily for 6 days. The patients were a 79 kg man receiving 1 g of primidone plus 0.4 g of diphenylhydantoin daily and an 81 kg man receiving 2 g of primidone plus 0.3 g of diphenylhydantoin daily. The former patient had been receiving primidone for 3 weeks and the latter for 8 weeks. Both denied taking any drugs other than those prescribed, *viz.* primidone and diphenylhydantoin.

The methods used for measuring phenobarbital both in dog plasma and in human plasma are not subject to interference from unchanged primidone. In samples of plasma from both species it was shown that the substance determined as phenobarbital has spectra characteristic of that compound both in the univalent anionic form and the bivalent anionic form. When extracts of human plasma were passed through the Craig countercurrent distribution train, the material determined as phenobarbital appeared in maximal amount in the nineteenth effluent fraction, the same fraction in which phenobarbital is in maximal amount. From the tenth to the thirtieth fraction, the amount of the material determined as phenobarbital conformed closely to that expected for pure phenobarbital. Thus in the fractions in this range the material determined as phenobarbital behaves as a homogeneous substance with a partition coefficient between the organic solvent and the buffer identical to that of phenobarbital. The evidence seems conclusive that phenobarbital is present in plasma after the administration of primidone and that the analytical methods used have not measured a significant amount of any substance other than phenobarbital.

In the dogs receiving primidone for 6 days there was progressive accumulation of phenobarbital in the plasma, the highest concentration being reached on the day after dosage

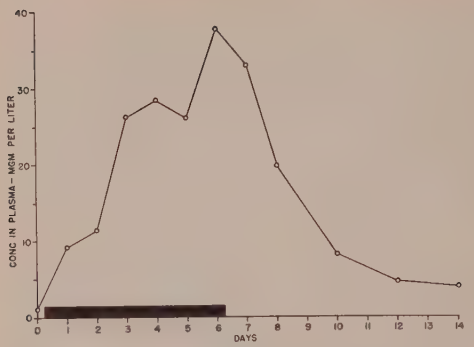

FIG. 1. Plasma phenobarbital concentrations resulting from oral administration of 200 mg/kg of primidone daily for 6 days to a 9.7 kg female dog. Period of drug administration is indicated by the bar.

was discontinued. The peak concentration was 27 mg/l in the dog on a daily dose of 250 mg/kg and 38 mg/l in the dog on a daily dose of 200 mg/kg. Fig. 1 shows the course of accumulation and elimination of phenobarbital in the latter dog.

In the patient receiving 1.0 g of primidone daily the plasma concentration of phenobarbital was 16 mg/l. In the patient receiving a daily dose of 2.0 g the plasma concentration of phenobarbital was 39 mg/l.

Discussion. The isolation of phenobarbital and its hydroxy derivative from urine and the demonstration of a substance in plasma with distribution coefficient and absorption spectra characteristic of phenobarbital give unequivocal evidence that primidone is in part converted to phenobarbital. During the course of this investigation it came to our attention that G. L. Plaa, J. M. Fujimoto, and C. H. Hine at the University of California, San Francisco, had independently found evidence of the presence of a substance with the spectral characteristics of phenobarbital in the blood of patients and in the urine of rats receiving primidone.

Comparison of the plasma concentrations of phenobarbital resulting from the administration of primidone and of phenobarbital itself permits a rough estimate of the proportion of primidone converted to phenobarbital. Calculations based on the phenobarbital concentrations found in other dogs and other patients receiving phenobarbital in chronic schedules lead to the conclusion that the dogs of the present study converted of the order of 5% and the patients of the order of 15% of the administered dose of primidone to phenobarbital. Thus the extent of the conversion is only small; but owing to the high dosage in which primidone is used and the slow rate at which phenobarbital is eliminated, that product may accumulate to reach levels sufficient to exert a significant effect. The concentrations of phenobarbital found in the plasma of the two patients of this study are in the range of those found in patients treated with antiepileptic doses of phenobarbital itself. There can be no doubt that concentrations of phenobarbital as high as those present in these two patients can be effective in suppressing seizures even in the absence of other drugs. While it seems clear that phenobarbital must, in some patients at least, make a significant contribution to the therapeutic effects of primidone, the role of the unchanged drug is not clear. If, as has been reported, seizures can be controlled in some patients by primidone and not by phenobarbital alone, the therapeutic results in such patients cannot be attributed entirely to the phenobarbital produced by the oxidation of primidone.

Summary. Primidone (Mysoline) is in part converted to phenobarbital by dog and by man. Concentrations of phenobarbital found in plasma of patients receiving therapeutic doses of primidone are high enough to exert a significant antiepileptic effect.

We are indebted to Dorothy Johnson for technical assistance and to Dr. Thomas W. Farmer for making patients available for some of these studies.

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Received October 8, 1956. P.S.E.B.M., 1956, v93.

Reaction of Viral Hepatitis Sera with *M. rhesus* Erythrocytes.* (22814)

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Latent or asymptomatic hepatic disease has been demonstrated in apparently healthy blood donors, and has been shown to be transmissible(1). In a recent study(2) 3655 apparently healthy blood donors were given a series of hepatic tests. According to the authors' evaluation 29% of these showed abnormalities, 17% "moderate" and 12% "severe." The authors concluded there was evidence that some, but not all, asymptomatic carriers will be excluded by the "hepatic test" screen, but that elimination of so large a fraction of blood donors would create a serious problem in supplying donor blood. It is generally agreed that some type of screening is needed, and that absence of jaundice during a number of months preceding blood donation does not insure freedom from infectivity.

Heterospecific serological tests have been found very helpful in the laboratory study of infectious diseases; agglutination of sheep erythrocytes in serum from patients with infectious mononucleosis has become a definitive test in diagnosing this condition. This suggested the possibility that a similar test might be found which would aid in the detection of viral hepatitis. Accordingly, sera from individuals diagnosed as having viral hepatitis were tested for their ability to agglutinate red cells of mouse, sheep, dog, guinea pig, steer, chicken, rabbit, horse, and So. American ring-tailed monkey, with no evidence that the cells could yield useful information. However, when red cells of rhesus monkey were used, agglutination was observed to a degree which was noticeably greater than was the sera from random controls. Erythrocytes from this species were therefore chosen for more intensive study.

Methods. *Macacus rhesus* red blood cells were collected in A. C. D. solution and stored in the refrigerator. A working suspension was prepared by washing the cells 3 times in 0.9% NaCl solution, resuspending them after the last washing to make up a 2% suspension by volume. The working suspension is made up just before using, and unused portions are discarded. The cells stored in A. C. D. solution gradually lose their sensitivity and should not be used after 10 days' storage. Sera to be tested were inactivated at 56C° for 30 minutes. 0.2 ml of serum was added to 0.2 ml of 0.9% NaCl solution and mixed; 0.2 ml of this dilution was transferred serially through 6 tubes, each containing 0.2 ml of diluent, giving a series of dilutions ranging from 1:2 to 1:64. 0.2 ml of cell suspension was added to each tube and the set was incubated for 1 hour at 37°C. Cells were sedimented by slow centrifugation (1000 r.p.m. for 2 min.) and degree of agglutination was estimated after gentle resuspension. The end-point was considered to be the tube containing the highest dilution of serum in which a definite agglutination was macroscopically visible. One of the problems in this kind of study is selection of a control group which may be presumed to be free from false positive reactions. In this case we chose sera from the pilot tubes of bank blood, since donors are questioned as to possible attacks of jaundice in the past, and must conform to certain physical standards indicating their apparent healthy condition. Even so, we must assume that some of these may be asymptomatic carriers of one of the hepatitis viruses, so the occurrence of positive reactions in this group cannot be considered as being false positives. In such a situation where control and experimental groups may overlap it is necessary to rely upon the differences in distribution of the results rather than upon absence of any positive reactions in the control group.

In addition to the control group, 3 others were studied. These included a group of patients diagnosed as having viral hepatitis,

* Appreciation is expressed for the assistance of Lucille Raitch, and Monica Stevens.

TABLE I. Agglutination Titers against Rhesus Erythrocytes.

Group	Highest serum dilution giving a positive test: Under 1:2	1:2	1:4	1:8	1:16	1:32	Total
I. Blood donors	65	18	27	12	3	0	125
II. Jaundiced, exclusive of viral hepatitis	8	4	2	0	0	0	14
III. Infectious mononucleosis	2	5	1	4	3	0	15
IV. Viral hepatitis	0	2	3	8	5	1	19

another group of jaundiced patients whose liver pathology was due to extrahepatic obstruction, Laennec's cirrhosis or malignancy, and a third group of individuals whose illness was diagnosed as infectious mononucleosis.

Results. The results of the agglutination tests are listed in Table I and presented graphically in Fig. 1. Table I shows that distribution of the titers in the second group (patients whose icterus was not due to viral hepatitis) did not differ significantly from the blood donor group. Therefore these two were combined in Fig. 1 to form the control group. Sera in this group were found to have comparatively low titers, 124 of the 139 specimens (89%) being less than 1:8. On the other hand, sera of the viral hepatitis group showed higher titers, 14 of the 19 specimens being positive in dilutions of 1:8 or more. The infectious mononucleosis sera were less uniformly distributed; the possible significance of this is discussed below. Table II compares the distribution of the titers observed in 19 hepatitis patients with the distribution which might have been predicted from the frequencies found in the control group.

Discussion. These observations indicate that one of the results of viral hepatitis is an increase in the ability of the patient's serum to agglutinate the red cells of the rhesus monkey. We are not able to say whether this effect is due to the presence of a true antibody, to the presence of the virus itself, or whether the reaction is based upon the appearance of an abnormal serum constituent resulting from physiological disturbances related to the disease. A test such as we have described, if proven to be reliable, should have two important functions. First, it would be a valuable aid in the differential diagnosis of the jaundiced patient. In our limited series the individuals whose jaundice was related to hepatic disease other than viral hepatitis fell into the normal category insofar as the rhesus erythrocyte agglutina-

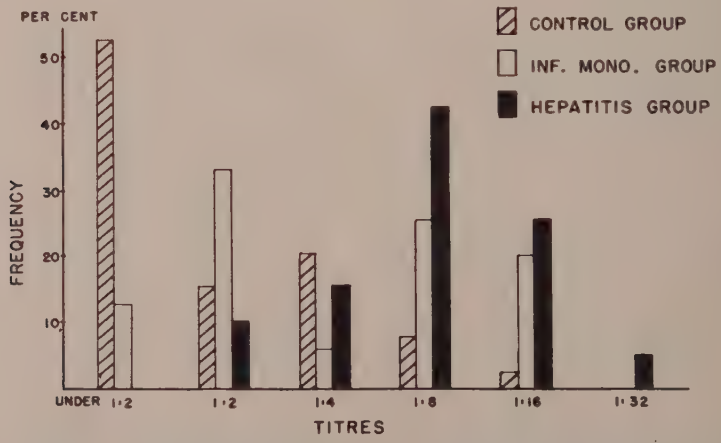

FIG. 1. Comparison of titres observed in control, infectious mononucleosis and viral hepatitis groups. The values represent the percentages of each group having the indicated titres.

TABLE II. Comparison of Expected and Observed Distribution of Titers in the Hepatitis Group.

	Titers					
	Under 1:2	1:2	1:4	1:8	1:16	1:32
Expected frequency based on control group	10	3.1	3.9	1.6	0.4	0
Observed frequency in hepatitis group	0	2	3	8	5	1

tion is concerned. Of the 19 cases of viral hepatitis, 14 showed elevated agglutinations titers. Thus, an agglutination titer of 1:8 or greater would support the diagnosis of viral hepatitis, while a titer less than 1:8 would argue against such a possibility. Since the test is positive (a titer of 1:8 or more) in almost one half of our cases of infectious mononucleosis. it cannot be said that it is a specific test for viral hepatitis. However, the liver is sometimes involved in infectious mononucleosis, so the positive tests obtained here may be related to hepatic disturbance. Furthermore, since infectious mononucleosis is ordinarily recognized by hematological and serological tests, the value of the rhesus erythrocyte agglutination reaction is not decreased by this finding.

A second possible field for use lies in the screening of donors to be used in the collection of whole blood or blood plasma. If the test were applied to the donors reported here, 15 of the 125 examined would have been rejected as possible asymptomatic carriers of viral hepatitis. Of course the evidence is purely presumptive, and further studies may show that such individuals may in fact not be carriers. However, since a substantial percentage of infected individuals in the viral hepatitis group (Gr. IV.) is detected by the test, its use as a screening device should be investigated. The fact that some infectious mononucleosis sera give positive reactions does not detract from its possible value as a screening procedure since it may be equally desirable to eliminate these individuals from the donor groups.

Summary. A hemagglutination test is described, wherein the sera of individuals diagnosed as having contracted viral hepatitis agglutinate the red blood cells of the rhesus monkey in dilutions of 1:8 or greater, as contrasted with the sera of a majority of apparently healthy blood donors and of jaundiced patients in whom a diagnosis of viral hepatitis was excluded. The observation, although made empirically and requiring confirmation, appears to offer promise and is being investigated at greater lengths.

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Received October 15, 1956. P.S.E.B.M., 1956, v93.

Hypotensive Activity of Natural and Synthetic Estrogens in Metacorticoid Hypertensive Rats. (22815)

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Estrogens were reported some years ago to have a hypertensive action in rats(1,2); however, subsequent workers were unable to confirm such effects on the blood pressure of normal or renal hypertensive animals(3-7). On the contrary, several reports suggested an antihypertensive action of estrogens(8-10). The present paper confirms the latter in the case of the metacorticoid hypertensive rat treated with 3 natural and 2 synthetic estrogens.

Methods. The experimental animals consisted of 80 male, Sprague-Dawley rats that had received subcutaneously a 40 mg wax pellet containing 20 mg desoxycorticosterone acetate (DCA) at least 3 months previously and that had been maintained on 0.86% NaCl solution and Rockland Rat Diet *ad libitum*. Saline and food were not removed during the course of the tests. The systolic blood pressures of treated rats were estimated without anesthesia or heating using a photoelectric tensometer(11) before and 2, 4, and 6 hours after injection, and were compared to simultaneous readings from solvent-injected controls. Pressure readings on individual rats were made in ignorance of the injection the rat had received and of the previous pressure readings. Each test usually consisted of 4 treated and 4 control rats run simultaneously by one person on the same tensometer in a darkened room. The 5 estrogens listed in Table I were injected subcutaneously in a total of 40 rats as 1% solutions in corn oil at a dosage of 20 mg/kg. Injected material was always deposited at least 60 mm from the site of puncture in order to prevent leakage. The significance of *changes* in blood pressure, as compared to those in the controls, was estimated by the rank-sum method(12).

Results. The mean group blood pressures are listed in Table I. The 3 natural estrogens had a mild anti-hypertensive effect, while the 2 synthetic compounds were more potent. The fall in blood pressure after treatment with the epiestriol derivative was highly significant and reduced blood pressure to the normotensive range. From the subsequent condition of the animals and also from our experience with many other steroids, there was no reason to ascribe the hypotensive responses to a general toxic action.

The 3 natural estrogens were considerably more potent than the 2 synthetic compounds with regard to their metrotrophic, osteotrophic, and lipemic actions, as measured by uterine growth in immature mice(13), bone density in adult mice, and blood lipids in cholesterol-fed chicks, respectively.* This is in contrast to the greater hypotensive activity of the synthetic estrogens (Table I).

Summary. Three natural and 2 synthetic estrogens were compared for hypotensive activity in metacorticoid hypertensive rats. The most active compound was 16-epiestriol 3-methyl ether.

TABLE I. Effects of Estrogens in Metacorticoid Rats.

Compound	No. rats	Mean group blood pressure, mm Hg			
		Before inj.	2 hr	4 hr	6 hr
Estrone	4	188	178	176	170*
Estradiol	8	185	172	173	164*
Estriol	8	171	174	163†	160*
16-epiestriol 3-methyl ether	8	186	143‡	139‡	130‡
16-oxoestradiol 3-methyl ether	12	180	165	162*	154†
Pooled controls	40	187	183	188	188

* $P < .05$. † $P < .01$. ‡ $P < .001$.

* To be published by R. A. Edgren and D. L. Cook.

I am grateful to Elizabeth Bosacki and Esther Lacunza for their valuable technical assistance and to Dr. David A. Tyner of the Division of Chemical Research for making these compounds available.

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Received August 27, 1956. P.S.E.B.M., 1956, v93.

Some Aspects of Relationship between Antigens of *Pasteurella pestis* and *Pasteurella pseudotuberculosis.* (22816)

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Schütze(1) established that *Pasteurella pseudotuberculosis* and *P. pestis* shared a common somatic antigen. Bhagavan *et al.* (2) have recently confirmed Schütze's finding and have presented data indicating that 5 of 7 *P. pestis* antigens are shared with *P. pseudotuberculosis*. Fraction I of *P. pestis*, according to the authors, is not shared with *P. pseudotuberculosis*. Since the toxin of the plague bacillus is considered an endotoxin, as originally stated by Rowland(3), it can be postulated that as an antigen it would be located in the somatic complex. The study of plague toxin has, in fact, usually been preceded by some operation designed to rupture the cells, such as prolonged growth followed by treatment with toluene(4), alternate freezing and thawing(5,6), and neutral salt extraction of acetone-killed bacilli(7). Somatic antigens of *P. pestis* which are shared with *P. pseudotuberculosis* do not include the plague toxin (2,8,9). For this reason, the toxin of *P. pestis* may be defined as a somatic antigen which is not shared with *P. pseudotuberculosis*.

The present communication describes attempts to establish these criteria using a previously described gel-precipitin technic(10, 11) by comparing lysed and unlysed suspensions of *P. pestis* and lysed suspensions of *P. pestis* and *P. pseudotuberculosis*.

Methods and materials. (a) *Preparation of cell suspensions*: Organisms were grown on heart infusion agar (Difco) at 37°C and harvested in phosphate buffered saline at pH 7.0. Organisms used were *P. pestis* strain A1122 and *P. pseudotuberculosis* strain 1, received from Dr. S. F. Quan, Communicable Diseases Laboratory, USPHS, San Francisco, Calif. The final suspensions contained 1.97-2.00 mg total nitrogen/ml. (b) *Rupture of cells*: Suspensions described above were placed in the glass receiver tubes of the Mickle disintegrator(12). Approximately 5 g of "ballottini"(12) were added to 20-30 ml of organism suspension. The instrument was allowed to vibrate at its maximum amplitude for 15 minutes (time determined by preliminary trials to be described in the next section. (c) *Determination of patterns in 2-channel comparator cells*: The technic used was similar to that described previously(10,11), using 1:50 anti-plague serum globulin in 1% clarified agar as internal reactant(13). *Experi-*

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Fig. 1

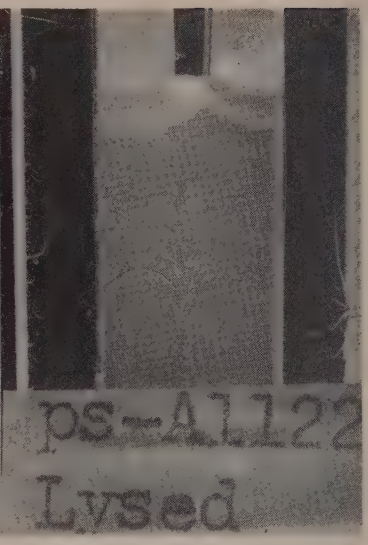

Fig. 2

FIG. 1. Antigen comparator cell showing match between a lysed suspension of *Pasteurella pestis* strain A1122 (on the left) and an unlysed suspension.

FIG. 2. Antigen comparator cell showing a match between lysed suspensions of *P. pseudotuberculosis* (left) and *P. pestis* strain A1122 (right). Approximately 2× enlargement.

mental observation. Three types of experiments were carried out and will be reported in this paper: (1) Lysis of suspensions of plague bacilli; (2) Comparison of lysed and unlysed suspensions with respect to antigenic components; (3) Comparison of lysed suspensions of *P. pestis* with those of *P. pseudotuberculosis.*

(1) *Lysis of suspensions of plague bacilli*: As mentioned above, suspensions of *P. pestis* strain A1122 were subjected to high speed vibration in the Mickle disintegrator in the presence of very small glass beads (ballottini). At 10-minute intervals specimens were withdrawn, the turbidity noted, and added to an aliquot of formalinized saline. Direct smears and cell counts using the Petroff-Hausser counting chamber, were made on the formalinized suspensions. The smears were Gram stained, density of cells and morphological characteristics were evaluated qualitatively. In general, high speed vibration produced with time a diminution in cell count accompanied by a decrease in turbidity of the cell suspensions. Approximately 90% of the reduction in cell count occurred during the first 15-20 minutes of shaking; subsequently, little change took place. The data indicated that this was due to an inherent inefficiency of the process with respect to small coccoid forms. Cultures of plague bacilli are usually mixtures of these and of larger bacillary forms. The disappearance of the latter was readily followed on the Gram stained smears. The action of high frequency vibration was to destroy selectively the larger forms.

(2) *Antigenic comparison of lysed and unlysed suspensions*: The mixture obtained by lysis in the Mickle disintegrator for 15 minutes was matched in 2-channel comparator cells against a suspension of identical composition except that it was not lysed. The test was set up in triplicate. The results of all 3 tests were essentially as shown in Fig. 1. Three rapidly moving components which were common to both preparations produced precipitin zones at considerable distance from points of origin. The fastest and the slowest of these components were present in similar concentration in both lysed and unlysed suspensions, as judged by the distance of their

precipitin zones from the meniscus. Since the distance of diffusion was not increased by lysis, these zones are marked as being of extracellular origin. The third component in this group was of intermediate mobility with respect to the other two, and its distance from the origin was increased on the side of the lysed suspension. The result, as shown in Fig. 1, was a zone of sharp equivalence point characteristics which curved upward from left to right of the comparator cell. The next two zones were common to both suspensions, but significantly increased in concentration in the lysed suspension, as shown by the greater distance of movement on the left side of the cell in Fig. 1. This condition may be explained by postulating 2 endo-antigens which are present in the intercellular fluid due to leakage or autolysis of some cells, but are markedly increased in concentration when all cells are ruptured. Finally, a broad 6th zone proximating the meniscus is present only on the side delivering material from the lysed suspension. This component must be an endo-antigen present in significant concentration only when most of the cells are lysed.

(3) *Antigenic comparison of lysed suspensions of P. pestis and P. pseudotuberculosis*: Lysed suspensions of *P. pestis* strain A1122 and *P. pseudotuberculosis* strain 1 were matched in 2-channel comparator cells as before. After 10 days, the appearance was that shown in Fig. 2. Visual and photographic observations confirmed the fact that the zones produced by the lysed *P. pestis* suspensions were identical to those produced in the preceding test, except for the presence of 3 instead of 2 zones in the intracellular antigen complex. (a) Two extracellular antigens appeared to be shared by both bacterial populations. These are the most rapidly moving zones which were present in lysed and unlysed *P. pestis* suspensions in like concentration. The third extracellular antigen of *P. pestis* was not shared with *P. pseudotuberculosis,* as indicated by the semi-radial pattern arising from the right (*P. pestis*) side of the cell. This precipitation zone had the same characteristics with respect to density as the antigen whose mobility was increased by lysis in the preceding test. (b) The next two intracellular antigens of *P. pestis* were not shared with *P. pseudotuberculosis.* (c) Finally, there was striking evidence that the slowly diffusing meniscus antigen of *P. pestis* is present in greater concentration in *P. pseudotuberculosis.*

Discussion. The finding of 7 antigens of *Pasteurella pestis* in the avirulent strain A1122 confirms the findings of Bhagavan *et al.* (2). However, it must be emphasized that this pattern does not represent a maximum or fixed number of possible components. Many of the variables involved have been mentioned by Oudin(14), Munoz and Becker (16), Jennings(15), and Ransom *et al.*(11).

The following description appears from this work to represent essentially the antigenic structure of *P. pestis* and *P. pseudotuberculosis.* There are at least 2 antigens common to both organisms which diffuse readily from the surface of the cell, which probably correspond to the "envelope" antigens of Schütze (loc. cit.). A third *P. pestis* antigen which diffuses quite readily from suspensions but is increased in concentration when the cells are disintegrated is not shared with *P. pseudotuberculosis.*

The finding of 2 shared "envelope" antigens between these two organisms confirms the observation of Larson *et al.*(17) that cross reacting antigens are present in ether-treated water-soluble preparations of the two organisms. Since Fraction I has been shown to be absent in *P. pseudotuberculosis,* this antigen is probably represented by the third zone of intermediate mobility referred to above.

There are 2 or 3 antigens which are so intimately associated with the soma that their appearance in the extracellular fluid is limited to relatively small quantities unless the cell is broken up. These *P. pestis* antigens are not shared with *P. pseudotuberculosis.*

There is at least one intracellular antigen which is so firmly bound to the soma that it does not appear in these tests at all unless the cell walls are very largely destroyed. This antigen is possessed also by *P. pseudotuberculosis,* and is perhaps identical with the so-

TABLE I. Distribution of Antigens in *Pasteurella pestis* and *P. pseudotuberculosis* as Revealed by the 2-Channel Comparator Cell.

Probable cellular location	Shared	*P. pestis* only	*P. pseudotuberculosis* only
Envelope	2	1*	0
Somatic	0	3†	0
Intracellular	1	0	0

* Fraction I? † Fraction II?

matic antigen of Schütze.

Since toxin is possessed by *P. pestis,* including strain A1122, but not by *P. pseudotuberculosis,* one of the unshared antigens most probably represents the toxin. Plague toxin is known to be firmly bound to the cell. The only likely candidates then, assuming that plague toxin is represented in these tests by one or more precipitation zones, are the two or three somatic antigens which *P. pestis* does not share with *P. pseudotuberculosis.* The hypothetical distribution of antigens between *P. pestis* and *P. pseudotuberculosis* is shown in Table I.

Summary. It has been shown that high speed vibration of cells of *P. pestis* in the presence of "ballottini" is accompanied by disintegration of the bacillary forms with concurrent release of antigenic material. At least one antigen was present in the lysed material which could not be demonstrated when cells were not lysed. 2. *P. pestis* and *P. pseudotuberculosis* appear to share 2 free "envelope" antigens as well as a single somatic antigen. In addition, it has been shown that *P. pestis* possesses a minimum of 1 "envelope" and 3 somatic antigens which are not shared with *P. pseudotuberculosis.* No antigens which could be considered specific for *P. pseudotuberculosis* were revealed.

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Received August 27, 1956. P.S.E.B.M., 1956, v93.

Comparison of Noludar (Methylprylon) and Pentobarbital on Respiratory Minute Volume in Rabbits.*† (22817)

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Noludar (3,3-diethyl-2, 4-dioxo-5-methyl piperidine),‡ has been demonstrated in both animal and clinical trials to be a mild hypnotic and sedative with very few side effects (1,2). It has been suggested clinically that Noludar, when given with morphine, does not add to respiratory depression produced by the opiate and hence would be a desirable agent to be used when combination therapy is necessary.§ The purpose of this study was to compare respiratory depressant effects of Noludar and pentobarbital alone and in combination with morphine on rabbit respiratory minute volume.

Methods. Rabbits, ranging in weight from 1 to 2 kg, were used. Respiratory minute volumes were determined in 2 L. spirometer which collected expired air by means of a Lucite mask fitted with one-way flutter valves. In each experiment, at least 3 control values were determined before injection of the drug. Following control determinations, the drugs, dissolved in normal saline, were injected into marginal ear vein. When morphine was used it was always injected before the hypnotic drug. Minute volume measurements were then taken every 15 minutes for a 2-hour period after injection. Respiratory minute volume depression was expressed as a percentage of control and these percentages summed for the 2-hour period to yield 2-hour total area values(3). All comparisons were made from these values.

Results. Fig. 1 represents the calculated regression plots for morphine (M), Noludar (N), pentobarbital (P) and combinations of

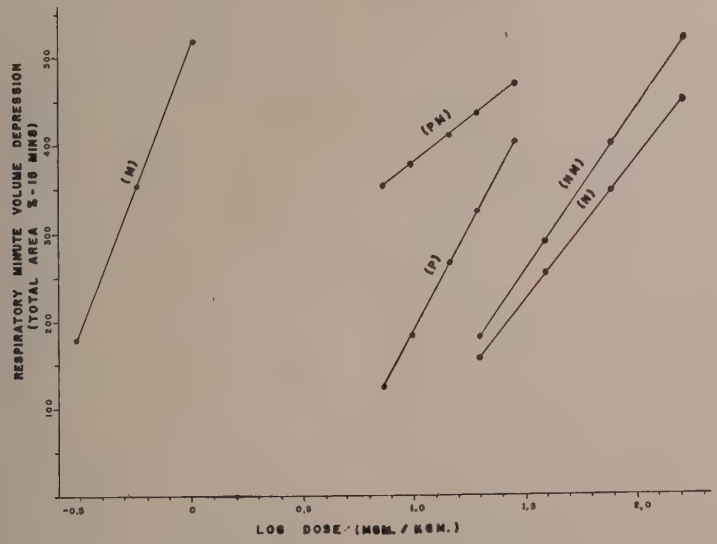

FIG. 1. Calculated regression plots for M (morphine), P (pentobarbital), N (Noludar), NM (Noludar-morphine) and PM (pentobarbital-morphine).

* This material to be submitted to Graduate College of State University of Iowa by H. C. Stanton in partial fulfillment of requirements for degree of Doctor of Philosophy.

† This research was supported in part by grant from Hoffmann-LaRoche, Inc., Nutley.

‡ We wish to thank Dr. M. J. Schiffrin for the Noludar.

§ Unpublished communication.

TABLE I. Regression Statistics for Regression Lines Fig. 1.

Drugs	No. animals/regression line	a*	b	$\bar{x}$†	Linearity of regression F	Significance P
Morphine (M)	18	360.56	566.12	.199	54.66	.001
Pentobarbital (P)	25	271.16	471.35	1.19	27.82	"
Noludar (N)	26	269.62	318.77	1.66	55.53	"
Morphine-pentobarbital (MP)	25	411.64	191.75	1.18	6.66	.05–.01
Morphine-Noludar (MN)	22	336.37	364.91	1.73	35.83	.001

* % total area (15 min. for 2 hr). † Log dose.

Regression equation, $Y = a + b\,(x - \bar{x})$.

pentobarbital (PM) and Noludar (NM) with 0.608 mg/kg of morphine. The data in each case were tested for linearity of regression and in all cases were found to be significantly linear. These regression statistics (4) are collected in Table I.

Comparisons of slopes of regression lines were conducted by means of t test of regression coefficients. Morphine had a significantly greater slope than Noludar ($t_{(42)} = 0.01 > p > 0.001$), than pentobarbital-morphine combination ($t_{(39)} = 3.50 p < 0.001$) and the Noludar - morphine combination ($t_{(36)} = 2.00$ $0.05 > p > 0.02$). The slope of pentobarbital alone was greater than that of pentobarbital-morphine combination ($t_{(46)} = 2.41$ $0.02 > p > 0.01$). Morphine when compared to pentobarbital and Noludar when compared to pentobarbital or Noludar-morphine were not found to be significantly different. The slope of Noludar-morphine, although somewhat greater than that of pentobarbital-morphine, was not significantly greater ($t_{(43)} = 1.80$ $0.10 > p > 0.05$).

The differences in slope among the various drugs and combinations do not allow direct comparisons of potency. We have therefore compared the differences between low and high doses of pentobarbital alone and with morphine and similarly low and high doses of Noludar alone and with morphine. These comparisons are detailed in Table II. Noludar-morphine combinations do not differ significantly from Noludar alone at either high or low doses. Pentobarbital-morphine combinations are, however, significantly more depressant than pentobarbital alone at low dose but not at high doses.

TABLE II. Comparison of Low (N–NM), High (N–NM), Low (P–PM) and High (P–PM).

Log dose (x)	Comparison	$t_{(df)}$	Significance P
.933	P_1–PM_1	$6.01_{(46)}$	.001
1.569	P_2–PM_2	$.74_{(46)}$	.50–.40
1.285	N_1–NM_1	$.58_{(44)}$	.60–.50
2.226	N_2–NM_2	$1.42_{(44)}$	.20–.10

TABLE III. Effect of Intermittent Injections of Pentobarbital and Noludar in Normal and Morphine-Treated Rabbits.

No. of doses of depressant	Morphine pretreatment, mg/kg	Noludar* treatment† effect
6	0	Survived
8	0	"
7	.608	Died 60 min. post-inj.
8	.608	Survived
4	1.220	"
7	1.220	"
8	1.220	Died 60 min. post-inj.

* All pentobarbital animals died at end of last dose.

† Each pentobarbital dose contained 10 mg/kg and each Noludar dose 35.5 mg/kg as calculated from regression lines as producing equivalent respiratory minute volume depression. Inj. rate was 1 dose/min.

In view of the apparent lack of difference between pentobarbital and Noludar at high doses, an attempt was made to compare pentobarbital and Noludar as to toxicity (*i.e.* cessation of respiration). Simultaneous intermittent injections of equidepressant doses of Noludar and pentobarbital were given to rabbits with and without morphine pretreatment. The results are summarized in Table III.

Discussion. There is some indication from the toxicity data that Noludar is less toxic than pentobarbital at equally depressant doses of the hypnotics alone and in combina-

tion with morphine. However, in evaluating these data, it must be considered that Noludar is a much longer acting hypnotic than pentobarbital and hence discrepancies in the time necessary to produce respiratory arrest might be expected. Nevertheless, 4 out of 7 animals survived for 4 hours or longer following Noludar while all 7 animals receiving equi-depressant doses of pentobarbital expired.

Summary. Noludar and pentobarbital alone and in combination with morphine were compared as to their respiratory minute volume depressant effects in rabbits. No statistically significant difference could be found between Noludar alone and in combination with 0.603 mg/kg of morphine. Pentobarbital, on the other hand, was significantly more depressant in combination with morphine at low pentobarbital doses but not at anesthetic levels.

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Received September 10, 1956. P.S.E.B.M., 1956, v93.

Carbohydrate Metabolism and Phosphate Turnover Rate in Scorbutic Guinea Pigs. (22818)

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Metabolism of carbohydrate is altered in ascorbic acid deficiency. Banerjee *et al.*(1-4) and Bacchus and Heiffer(5) observed decreased glucose tolerance in scorbutic guinea pigs. Murray *et al.*(6) and Stewart *et al.*(7) reported diminished glucose tolerance in scorbutic monkeys. Liver glycogen content was lower in such animals than in paired fed normal controls. Banerjee *et al.*(3,8,9) ascribed the decreased carbohydrate metabolism to diminished insulin content of the pancreas in scurvy. Recently Banerjee and Ghosh(10) reported diminished hepatic and muscle hexokinase activity in scorbutic guinea pigs possibly due to decreased insulin synthesis and supply in such animals. Insulin was shown to stimulate hexokinase(11) and reduce liver glucose-6-phosphatase activities(12). Considering the possible role of ascorbic acid in the biosynthesis of insulin and the functions of insulin at these levels of glucose metabolism, the glucose-6-phosphatase activity was determined in tissues of scorbutic and paired fed normal guinea pigs. Decrease in hexokinase activity and increase in glucose-6-phosphatase activity might consequently lead to diminished phosphorylated intermediates of the glycolytic system. The changes in phosphorylation and dephosphorylation processes might eventually alter inorganic phosphorus content of tissues in scurvy. Studies were, therefore, carried out to elucidate the pattern of metabolic changes in relation with carbohydrate metabolism in scorbutic guinea pigs. The hexokinases are known to be sulfhydryl enzymes, and it is now well accepted that ascorbic acid protects and maintains the sulfhydryl groups of the enzymes(13). As an introduction to further study on the role of ascorbic acid in carbohydrate metabolism, fructokinase activity of the brain tissue of scorbutic and paired fed normal guinea pigs was determined.

Methods. Female well-growing guinea pigs of weights varying between 230 and 260 g were distributed into 16 pairs. Feeding a scorbutic diet, with or without supplement of ascorbic acid, by paired feeding technic and care of animals were the same as described previously(14). Pairs of animals were sac-

TABLE I. Effect of Scurvy on Inorganic Phosphorus Values of Blood and Liver of 10 Guinea Pigs in Each Series.

	mg P/100 ml blood	mg P/100 g liver
Normal	4.7 ± .25*	34.1 ± 2.1
Scorbutic	6.2 ± .50	46.1 ± 1.2
t	2.5	4.8

* Stand. error.

rificed after an over-night fast on 25th day of experiment, and inorganic phosphorus contents of blood and liver, glucose-6-phosphate, fructose-6-phosphate and fructose-1, 6-diphosphate contents of liver, glucose-6-phosphatase activity of liver and kidney and fructokinase activity of brain were determined. Total weights of livers of some pairs of guinea pigs were recorded and the values calculated per 100 g body weight. For estimation of *inorganic phosphorus,* blood was collected in an oxalated tube and 1 ml aliquot was analysed by the method of Fiske and SubbaRow (15). For liver inorganic phosphorus and phosphorylated intermediates, the tissue was immediately frozen after killing the animal and was extracted with ice cold 10% trichloroacetic acid. A portion of the extract was neutralized, in cold, to phenolphthalein and inorganic phosphate was precipitated with magnesia mixture over night and then estimated. Another aliquot was analysed for glucose-6-phosphate, fructose-6-phosphate and fructose-1, 6-diphosphate according to the methods described by LePage and Umbreit(16). Results are given in Tables I and II. For estimation of *glucose-6-phosphatase* and *fructokinase* activity, tissues were removed from body immediately after killing the animal and cooled in cracked ice for 3-5 minutes. Glucose-6-phosphatase activity of liver and kidney was assayed by the method of Swanson(17). The tissues were homogenized with exactly 3 volumes of ice-cold 0.25 M sucrose in an ice-cold all glass homogeniser. 0.1 ml of this homogenate, 0.1 ml of 0.04 M glucose-6-phosphate (pH 6.5) and 0.1 ml of 0.1 M citrate buffer of pH 6.5 were mixed in 1 cm diameter duplicate tubes, incubated for 30 minutes at 37° and the reaction terminated by addition of 0.3 ml of 10% trichloracetic acid. In control tubes, water was added in place of the substrate. The samples were diluted to 3 ml, centrifuged and analysed for inorganic phosphorus (15). Dry weights of tissues were obtained from 1 ml homogenate, less the weight of sucrose present. Inorganic phosphorus liberated by the enzymatic hydrolysis in one hour per mg dry tissue is presented in Table III.

TABLE II. Effect of Scurvy on Phosphorylated Intermediate Contents of Liver of Guinea Pigs (mg/100 g Fresh Liver). 6 animals/series.

	Glucose-6-phosphate	Fructose-6-phosphate	Fructose-1,6-diphosphate
Normal	1166 ± 41	17.0 ± 1.1	20.2 ± .97
Scorbutic	995 ± 44	11.1 ± 1.5	15.7 ± 1.0
t	2.7	3.2	3.1

TABLE III. Effect of Scurvy on Glucose-6-phosphatase Activity of Tissues of Guinea Pigs (γ P Liberated/mg Dry Tissue/ Hr). 8 animals/series.

	Liver	Kidney
Normal	27.4 ± 4.2	31.0 ± 5.5
Scorbutic	40.5 ± 4.5	36.8 ± 15.0
t	2.1	.7

For determination of *fructokinase activity,* brain tissue was homogenized in an ice-cold all glass homogeniser with 4 volumes of ice-cold 0.15 M potassium phosphate buffer of pH 7.5 containing 0.15 M potassium fluoride. Fructose phosphorylation studies were studied in an incubation medium proposed by Vestling *et al.*(18), using 1 ml portions. Incubation was carried out for 30 minutes at 37° and the reaction stopped by addition of 1 ml of 0.3 N barium hydroxide, followed by 1 ml of 5% zinc sulfate and water to 10 ml, mixed and filtered. Control experiments were conducted using complete medium with omission of adenosinetriphosphate. Fructose disappearance was measured by the method of Roe(19). 1 ml homogenate was dried at 105° to constant weight, and weight of salts deducted to get dry weight of tissue. Fructose disappeared/mg dry tissue/hour is presented in Table IV.

Results. A change in tissue phosphorus content in scurvy is apparent from Table I. Though there is a significant increase in inorganic phosphorus content of blood, only 5 pairs out of 10 studied show this change. A

TABLE IV. Effect of Scurvy on Fructokinase Activity of Brain of 10 Guinea Pigs per Series. (γ fructose disappeared/mg dry tissue/hr.)

Normal	Scorbutic	t
123.3 ± 12	100.5 ± 35	.6

significant increase in liver inorganic phosphorus content in scurvy was observed. This increased inorganic phosphorus level of blood and liver in scorbutic animals suggests that either the mechanism responsible for removal of free phosphate is inefficient, or that hydrolysing the organic phosphates, *i.e.*, phosphatase activity, is increased.

We reported that hepatic and muscle hexokinase activity is diminished in scorbutic guinea pigs(10). Table III shows that glucose-6-phosphatase activity of the hepatic tissue is increased in scurvy. These changes are those which would be expected if an insulin insufficiency occurred in such animals. As a consequence it is likely that inorganic phosphorus level of tissues will tend to increase and glucose-6-phosphate content of liver would diminish in scurvy. The data presented in Table II clearly indicate that this ester content of livers of scorbutic guinea pigs is significantly decreased. This table further shows that fructose-6-phosphate and fructose-1, 6-diphosphate levels of livers in scurvy are lowered.

Table III shows that the hepatic glucose-6-phosphatase activity is increased in scurvy, and remains unchanged in the kidney. Langdon and Weakley(12) reported that alloxan diabetes in rats is accompanied by a more than 2-fold increase in hepatic content of the enzyme. In scurvy, however, there is comparatively less change, which could be due to a smaller decrease in insulin synthesis in scurvy than in alloxan diabetes. These findings, therefore, suggest that the quantities of phosphorylated intermediates turned over in unit time are reduced below normal and further add to the evidence that the defect in carbohydrate metabolism in scurvy is quantitative rather than qualitative. It is, however, difficult to assess whether these quantitative changes in the phosphorylation cycle are of sufficient magnitude to account for the disturbed glucose tolerance in scorbutic animals.

No change in fructokinase activity in the brain of guinea pigs has been observed in scurvy. Table IV shows that, though there is a tendency to decrease, in some scorbutic animals, the individual variations are too great to make the change in average values significant. Fructose phosphorylation is also not hampered in insulin insufficiency due to alloxan diabetes(19-21). It has been observed that in scorbutic guinea pigs the weights of livers were considerably increased. The values calculated in g/100 g body weight of normal and scorbutic guinea pigs are 2.8 ± 0.2 and 4.8 ± 0.4 respectively. If the inorganic phosphorus and glucose-6-phosphatase activity of livers of scorbutic guinea pigs is expressed on the basis of total liver, these values will be further increased.

Summary. Inorganic phosphorus content of blood and liver and hepatic glucose-6-phosphatase activity are increased in scorbutic guinea pigs. Glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate contents of liver are decreased in scorbutic guinea pigs. Fructokinase activity of brain remains unchanged in scorbutic guinea pigs. Considerable increase in weight of livers of scorbutic guinea pigs has been observed.

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Received September 13, 1956. P.S.E.B.M., 1956, v93.

Enzymatic Estimation of Urinary Steroids.* (22819)

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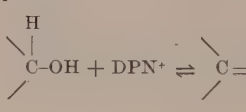
Existing procedures for the determination of urinary steroids depend upon either chemical reactions or bioassay. Despite great interest in this subject and its clinical importance, simple and specific micro-methods for the estimation of the amounts and types of urinary steroids have not been perfected(1). The novel procedure to be described here permits the simple microestimation of certain types of steroids in urine. This procedure depends upon selective enzymatic oxidations and reductions of steroids and the spectrophotometric measurement of the associated changes in the concentrations of diphosphopyridine nucleotide coenzymes. Enzymatic estimations are characterized by a high degree of specificity and sensitivity. It is thus possible to obtain quantitative information on the principal types of steroid ketones and alcohols present in a few milliliters of urine without resorting to tedious isolation and separation techniques. *Pseudomonas testosteroni* is a soil bacterium capable of growing on testosterone and related steroids as its only carbon source, and of oxidizing such steroids to carbon dioxide and water(2). The presence of steroids in the growth medium of this bacterium causes the induction of certain diphosphopyridine nucleotide-linked enzymes which reversibly interconvert particular hydroxy- and ketosteroids with a high degree of steric specificity(2-4). These enzymes have been named *hydroxysteroid dehydrogenases,* and their potential usefulness for the micro-estimation of steroids was pointed out in 1952. Subsequently, steroid dehydrogenases have been applied to the measurement and identification of single steroids(2-5). The purification and substrate specificities of 2 enzymes suitable for analytical purposes have been described(5-7). Briefly stated, these enzymes are: 1) a *3α-hydroxysteroid dehydrogenase* (designated α enzyme) which catalyzes the reversible oxidation of 3α-hydroxysteroids of the C-19 and C-21 groups; and 2) a *3β- and 17β-hydroxysteroid dehydrogenase* (designated β enzyme) which catalyzes the reversible oxidations of 3β- and 17β-hydroxysteroids to their respective ketones. Thus, the specificity of these enzymes fortunately coincides with the principal types of urinary steroids *i.e.* 17-ketosteroids as well as 3α-hydroxy- and 3β-hydroxysteroids of both the C-19 and C-21 series. The *analytical procedure* depends upon interconversions of steroid alcohols and ketones according to the general equation:



* Supported by grants from Am. Cancer Society, upon recommendation of National Research Council Com. on Growth.

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when DPN^+ and DPNH refer to the oxidized and reduced forms of diphosphopyridine nucleotide respectively. DPNH has an absorption maximum ($\epsilon = 6{,}220$) at the wavelength of 340 mμ, where DPN^+ has negligible absorption. Thus, the increase in absorption at 340 mμ in the forward reaction is an accurate measure of the amount of DPNH produced and hence of the amount of steroid alcohol oxidized. Conversely, the decrease in absorption of DPNH in the *back* reaction is a measure of the amount of ketosteroid reduced. An accurate estimation of the total amounts of steroids present requires conditions under which the reaction equilibria are displaced either completely to the right (forward reaction) or to the left (back reaction), even in the presence of relatively large quantities of reaction products.

Quantitative conversions in the forward reaction were achieved by raising the pH to 9.5 and incorporating hydrazine as a ketone binding reagent into the reaction mixture. The back reaction is favored by lowering the pH, but only limited use can be made of this because of the instability of DPNH below pH 5.5. Complete reduction of ketosteroids to steroid alcohols was obtained if diphosphopyridine nucleotidase (DPN-ase) from zinc deficient *Neurospora*(9) was added to the reaction system. This enzyme destroys the DPN^+ formed during the back reaction by rupture of the nicotinamide-ribose bond of DPN^+, but does not attack DPNH.

Materials and methods. Extracts of human urine were prepared from 25 ml aliquots of 24 hour collections. Each aliquot was subjected to hydrolysis of steroid conjugates by incubation with beef liver β-glucuronidase (1000 units(10) per ml of urine) at pH 4.5 and 30° for 24 hours. Following extraction with three 15 ml portions of CH_2Cl_2, the aqueous residue was acidified to pH 1 with H_2SO_4 and further incubated at 30° for 24 hours. The residue was then reextracted with three 15 ml portions of CH_2Cl_2 and the combined solvent extracts evaporated to dryness. All evaporations were carried out in a rotating evaporator *in vacuo* below 45°. The residue was dissolved in methanol and passed over a 70 × 10 mm column of Amberlite MB 1, a strong base-strong acid, anion-cation mixed bed exchange resin obtainable from the Rohm and Haas Co., Philadelphia. The resin was well washed with water and methanol before use. The ion exchange resin quantitatively removes phenolic estrogens and eliminates other ionic constituents. The extract was evaporated, redissolved in 70% aqueous methanol and partitioned against *n*-hexane. The methanolic layer was evaporated to dryness and the residue dissolved in 0.50 ml methanol. Aliquots of this extract were then subjected to enzymatic reaction first with α enzyme and then with β enzyme in both forward and back reactions. The preparation of the enzymes has been described(7). All reactions were carried out in a Beckman DU spectrophotometer in cuvettes of 1 cm light path at approximately 25° and 340 mμ. The system for the *forward reaction* contained in a total volume of 3.0 ml: 100 μmoles of sodium pyrophosphate buffer pH 9.5, 0.5 μmole DPN^+, 1.0 millimole hydrazine sulfate (adjusted to approximately pH 9.5) and 0.025 to 0.075 ml of urine extracted in 0.1 ml CH_3OH. Initial optical density readings were taken against a control cuvette containing all ingredients except urine extract. 1000 units α enzyme in 0.01-0.02 ml were then added and readings continued until there was no further change in optical density for 15 minutes. 1000 units of β enzyme were then added and readings continued again to equilibrium. In the *back reaction*, the cuvettes contained: 100 μmoles orthophosphate buffer pH 5.5, 0.12 μmoles DPNH, 200 units DPN-ase and 0.025 to 0.050 ml of urine extract in 0.1 ml CH_3OH. Initial readings were taken at 340 mμ against a control cell containing no urine extract and no DPNH. An additional control containing only DPNH and enzymes was included, to compensate for small decreases in optical density of DPNH occurring during the course of the reaction. α and β enzymes (1000 units of each) were added successively to the reaction cuvettes and measurements made periodically until equilibrium was reached with each enzyme. The measurements were based

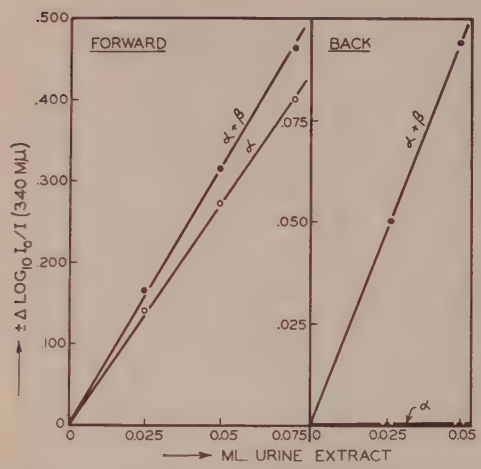

FIG. 1. Enzymatic assay of urinary steroid excretion in a 24-year-old healthy man whose output was 1350 ml in 24 hr. A 25 ml portion of urine was used to prepare 0.5 ml extract in CH_3OH as described in the text. Ordinate of the left graph shows the increases in optical density at equilibrium in the *forward reaction*, whereas that of the right graph shows the *back reaction*, in which decreases in optical density at equilibrium are plotted. Measurements were made with different amounts of urine extract and demonstrate linearity between the amount of pyridine nucleotide reduced or oxidized and vol of urine extract.

on the changes in optical density when each enzymatic reaction was complete.

Results. Typical measurements on an extract of male urine are shown in Fig. 1. Preliminary values for the steroid excretion of a small number of young subjects believed to be in good health are shown in Table I. The reliability of the assay procedure was established by several criteria: 1) A linear relation between the amount of extract and the change in optical density may be seen in Fig. 1, and has been repeatedly observed. 2) Various pure steroids added to a urine extract have been quantitatively estimated with a high degree of accuracy. The results of a recovery experiment of this type are shown in Table II. Suitable aliquots of a hydrolyzed urine extract and of solutions of purified androsterone and *epi*androsterone were each assayed successively with α and β enzymes in the *forward* reaction. The respective amounts of hydroxysteroids found with each enzyme are shown in experiments 1, 2 and 3 of Table II. A mixture of androsterone and *epi*androsterone was then assayed in the same manner and agreed within 1 percent or less with the individual assays obtained for each steroid separately (experiment 4). Similar aliquots of androsterone and *epi*androsterone were then measured in the presence of the same aliquot of urine extract and here also the recovery was better than 1% (experiment 5). Similar recovery experiments utilizing the *back* reaction have shown a recovery of *epi*androsterone measured as a 17-ketosteroid of within 1 to 2%. These experiments demonstrate the high accuracy of the method and excellent recoveries of physiological amounts of added steroids. 3) Some of the most abundant urinary steroids were added to an aliquot of urine which was then

TABLE I. Urinary Steroid Excretion in Micromoles per 24 Hours in Normal Young Adults.

Steroid groups measured	Men	Women	Examples of principal types of compounds measured
3α-Hydroxyl	54.6 (43.3 to 70.7)	34.8 (16.4 to 61.6)	Androsterone Etiocholan-3α-ol-17-one Pregnane-3α,17α,21-triol-11,20-dione Pregnane-3α,11β,17α,21-tetrol-20-one
3β-Hydroxyl + (17β-Hydroxyl)	8.86 (5.20 to 13.7)	5.98 (2.61 to 8.05)	*Epi*androsterone Dehydro*epi*androsterone
3-Ketone	<.5	<.5	Androstane-3,17-dione Etiocholane-3,17-dione
17-Ketone	21.4 (17.9 to 25.5)	9.71 (4.67 to 15.0)	Androsterone Etiocholan-3α-ol-17-one *Epi*androsterone Dehydro*epi*androsterone 4-Androstene-3,17-dione

Avg values are based on determinations in 6 men (age range 22 to 33 years) and 6 women (age range 23 to 37 years). Figures in parentheses refer to ranges of values in each group.

TABLE II. Estimation of Pure Steroids in the Presence of a Urine Extract.

Exp. No.	Material*	ml	μmoles of hydroxysteroids α	β	α + β
1	Urine extract	.05	.0580	.00725	.0653
2	Androsterone	.02	.0237	.000	.0237
3	*Epi*androsterone	.02	.000	.0435	.0435
4	Androsterone + *Epi*and'st'one	.02 .02	.0237	.0430	.0667
5	.05 ml urine extract + .02 ml androsterone + .02 ml *epi*androsterone		.0813	.0498	.131

* Urine extract was prepared from male urine as described in text (except that 1 ml extract was equivalent to 15 ml urine). Androsterone (m.p. 184.1-185.2° corr.) solution contained 3.5 mg in 10 ml methanol. *Epi*androsterone solution (m.p. 173-175° corr.) contained 6.5 mg in 10 ml methanol. Steroids were weighed to ± 0.1 mg. Values in table indicate that steroids were 94 to 96% pure by enzymatic assay.

carried through the entire procedure of hydrolysis and preparation of an extract as described. By enzymatic assay the recovery of androsterone was 94.5%, and that of pregnane-3α,17α,21-triol-11, 20-dione was 93.5%. 4) Duplicate assays on different aliquots of the same urine agreed within 5% or better.

Discussion. Several advantages characterize these enzymatic methods as compared to chemical and bioassay procedures. Sensitivity and specificity are inherent in enzymatic assays. The measurements depend upon specific changes in light absorption caused by DPNH, a compound of known structure, spectral characteristics and extinction, and may therefore be related directly to micromolar concentrations. These changes are produced by addition of a few micrograms of enzyme protein, which have negligible absorption at 340 mμ, and which produce no unspecific absorption changes or "background colors" at this or other wavelengths. Furthermore, unlike many chemical methods such as the Zimmerman reaction, equal amounts of different steroids give equivalent extinctions when measured by these enzymatic methods.

The sensitivity of the assay system depends upon the size of the reaction volume and the sensitivity of the spectrophotometer. Using microcells and a Beckman DU Spectrophotometer, 0.2 to 0.5 μg of steroid may be assayed with accuracy of a few per cent in a reaction volume of 0.2 ml. This sensitivity approaches that required for the measurement of the small amounts of steroids present in other body fluids such as the blood.

It is difficult to make comparisons between the magnitudes of the values shown in Table I and those available from other methods, because comparable measurements are available only for 17-ketosteroids. The values for 17-ketosteroids in normal young subjects by the enzymatic method are about 6 mg per day for men and 3 mg per day for women (assuming an average molecular weight of about 290). The respective ranges reported in the literature are 3 to 22 mg and 6 to 25 mg respectively(11). The enzymatic values are therefore consistent with the lower values obtained by the Zimmerman reaction, which are believed by some authors to be the most reliable(1,11).

Summary. A novel method for the sensitive and specific microestimation of steroids has been described. This method depends upon the selective oxidation or reduction of respectively hydroxy- and ketosteroids by highly purified hydroxysteroid dehydrogenases of bacterial origin. The method permits the estimation of 3α-hydroxysteroids, 3β-(and 17β-) hydroxysteroids, 3-ketosteroids and 17-ketosteroids.

The authors are indebted to Warner-Chilcott Laboratories, New York, for supplying "Ketodase" (β-glucuronidase) and to Syntex S. A. Mexico, for gifts of various steroids.

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Received September 13, 1956. P.S.E.B.M., 1956, v93.

Trace Metal Requirements of Azotobacter.* (22820)

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Although metallic ion requirements for fixation of atmospheric nitrogen by the azotobacter have been studied for a number of years(1,2,3) an extension and reevaluation of the earlier work was thought worthwhile in light of recent advances in methodology of trace metal studies. These earlier studies established marked differences in response of both strains and species of the organism. Therefore, an added reason was to determine trace metal requirements of our strain *Azotobacter vinelandii* O which is used in many biochemical and bacteriological laboratories.

Materials and methods. *Azotobacter vinelandii* O was maintained and studied in Burk's sucrose mineral salts medium(4) in 500 ml Erlenmeyer shake flasks. The cultures were incubated at 30°C in Brunswick rotary shaker at 400 rpm. Flasks were covered with 50 ml pyrex beakers instead of cotton plugs. All glassware was cleaned by washing with detergent followed by 4 rinses in distilled water. It was then filled with 0.5% solution of tetra sodium salt of ethylene diamine tetraacetic acid (Sequestrene Na 4, Alrose Chemical Co.) and autoclaved at 15 lb. pressure for 30 minutes. After autoclaving, glassware was rinsed 5 times with ion-free water obtained by passing distilled water through a Barnstead "Bantamac" demineralizer mode Bd-1. The effluent from the column contained less than .05 ppm total ionizable solids calculated as sodium ion equivalent. The 8-OH quinoline coprecipitation technic of Nicholas(5) was used to remove traces of iron and molybdenum from growth medium. This method was not suitable for investigation of calcium, so recrystallized analytical reagent chemicals were used. Nitrogen was determined by Kjeldahl semi-micro procedure(4). Turbidities were measured with Klett-Summerson colorimeter using the 660 mμ filter.

TABLE I. Effect of Nitrogen Source on Molybdenum Requirement of *Azotobacter vinelandii* O.

	μg N_2 fixed/ml*					
Exp.	N_2 .1 ppm Mo	N_2 –Mo	N_2 .2 ppm V	NH_4^+ .1 ppm Mo	NH_4^+ –Mo	Ashed NH_4^+
I				204	186	
II	205	50		201	200	
III		50			211	86
IV		56			211	
V	212	58		279	301	88
VI	262		83			
VII	166		62			

* In all tables, the ammonia grown cells harvested and washed with water, then resuspended in water and assayed for nitrogen. In all tables, "ashed NH_4^+" was prepared by heating ammonium acetate overnight in a porcelain crucible at 150°C. Residue was dissolved in dilute acid, neutralized and added to culture flasks.

Results. In agreement with previous studies with other strains, molybdenum and iron are both specifically required for nitrogen fixation process in *Azotobacter vinelandii* O. When ammonium ion is supplied as a nitrogen source the requirement is either eliminated, as with molybdenum or spared as with iron (Tables I and II). Vanadium has been reported to replace molybdenum in certain *Azotobacter* species(6) but this does not appear to be true with strain O.

* Supported by grants from Rockefeller Foundation and Research Committee of Graduate School from funds of Wisconsin Alumni Research Foundation.

TABLE II. Effect of Nitrogen Source on Iron Requirement of *Azotobacter vinelandii* O.

Added Fe^{+++} (ppm)	% maximum growth					
	N_2		NH_4^+		Ashed NH_4^+	
	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
1.0	100	100	100	100		
.5	92	83	100	99		
.1	59	51	86	77		
0	17	14	16	14	16	10

Species of *Azotobacter* grown on N_2 possess an active hydrogenase capable of carrying out the knallgas reaction(7), and H_2 has been shown to be competitive inhibitor of the fixation of nitrogen(8). The effect of various levels of iron and molybdenum on hydrogen inhibition of nitrogen fixation by *Azotobacter vinelandii* O was studied. The effect was studied in a sealed system employing a KOH trap to remove respiratory carbon dioxide output. Any net gas uptake in the growth flask was replaced with pure oxygen. The flasks were equipped with colorimeter tube sidearms so that Klett-Summerson readings could be made without disturbing the gas phase. The results of a typical experiment with iron are shown in Fig. 1. As can be seen the growth constant is drastically reduced in presence of H_2, independent of the level of iron (1-10 ppm) in the medium. However, an increase in iron content of the medium caused a large increase in the apparent oxygen uptake of the culture. Sucrose utilization and CO_2 evolution values were not sufficient to account for the increase in oxygen uptake, indicating that excess gas uptake was a product of a stimulated knallgas reaction. The effect of molybdenum was also studied over a range of 0.001 to 1.0 ppm and always a large increase in apparent oxygen uptake was obtained when hydrogen was present. The amount of increased oxygen uptake, however, was the same for the entire range of molybdenum concentrations tested.

Uncertainty exists as to the requirement of calcium for nitrogen fixation. For example, Horner and Burk(9) demonstrated what appeared to be a specific requirement in *Azotobacter vinelandii* but later(10) stated that the requirement for calcium was unchanged whether atmospheric nitrogen or ammonium ion was used as a nitrogen source. Jensen(11) has reported that calcium is not required by *Azotobacter indicum* but is required by *Azotobacter chroococcum*. The results summarized in Table III demonstrate a specific calcium requirement for nitrogen fixation by *Azotobacter vinelandii* O. The presence of ammonium ion apparently eliminates the need for added calcium. Strontium can replace calcium at approximately the same molar concentration. During this study it was noted that levels of calcium lower than 5 μg per ml in a nitrogen free medium resulted in an ex-

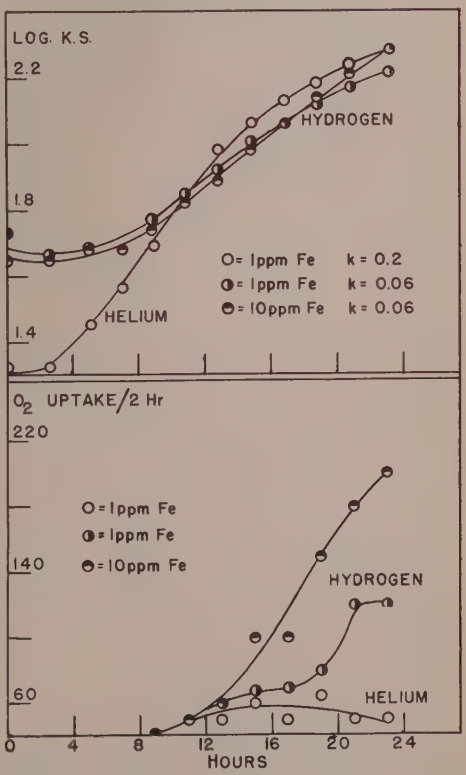

FIG. 1. Effect of iron conc. on hydrogen inhibition of nitrogen fixation by *Azotobacter vinelandii* O. k, velocity constant of growth $= \frac{2.3}{t_2 - t_1} \times \log \frac{KS_2}{KS_1}$ in which KS_1 is Klett-Summerson reading at time t_1, etc. Gas phase contained 0.2 atm O_2, 0.2 atm N_2 and 0.6 atm H_2 or He. Oxygen uptake in ml/flask/2 hr.

TABLE III. Effect of Nitrogen Source on Calcium Requirement of *Azotobacter vinelandii* O.

Added Ca^{++} (ppm)	μg N_2 fixed/ml		
	N_2	NH_4^+	Ashed NH_4^+
25	258	337	
10	241	308	
1	203	323	
.5	170	300	
0	13	313	26

tended lag period of growth followed by an apparently normal logarithmic phase. This is illustrated in Fig. 2. Varying the magnesium to calcium concentration ratio from 10 to 1 to 1 to 1 had no effect on the lag phase. There is an apparent interrelationship between calcium and phosphorus. Fig. 3 contains the results of an experiment demonstrating this interrelationship. Curves 1 and 2 show that phosphorus concentration does not effect the lag phase in the presence of an optimum amount of calcium. Curves 3 and 4 show that a low level of phosphorus

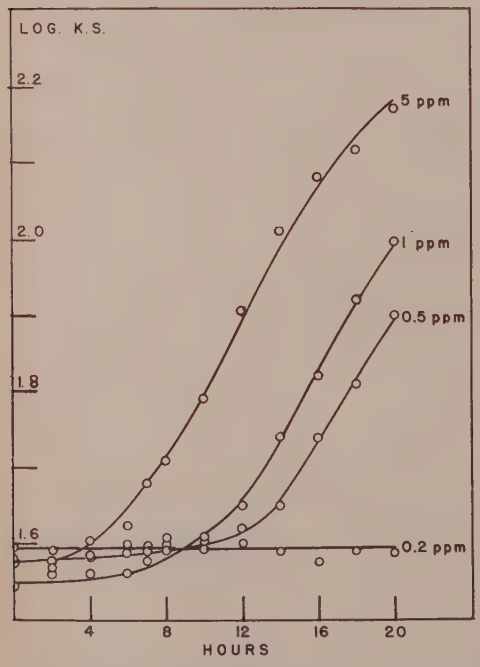

FIG. 2. Effect of calcium conc. (indicated ppm) on length of lag phase of *A. vinelandii* O fixing atmospheric nitrogen.

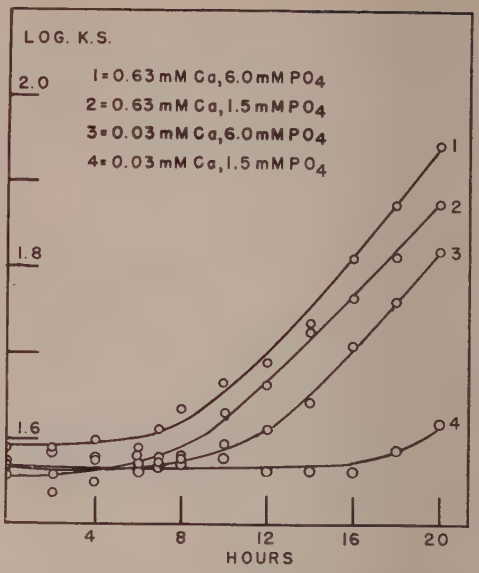

FIG. 3. Effect of varied calcium and phosphate conc. on lag phase of *A. vinelandii* O fixing atmospheric nitrogen.

results in a further lengthening of the lag phase of a culture with a suboptimal amount of calcium.

Discussion. The results of this study demonstrate that molybdenum, iron and calcium are specifically required for nitrogen fixation by *Azotobacter vinelandii* O, but the definite function of these metals in the fixation process is still to be established. However, molybdenum has been found to be a part of the hydrogenase enzyme system(12), and spectrophotometric studies of the effect of nitrogen and hydrogen on cell free preparations of nitrogen fixing organisms provide a function for iron via a hematin compound involved in the fixation process(13). The role of calcium in the system is even more obscure. The data reported here suggest that calcium functions in a reaction active during the lag phase, a reaction in which phosphate is also involved. The results with ammonium ion as a source of nitrogen indicate that the reaction or its products are not essential for the elimination of the lag phase unless the organism is actively fixing atmospheric nitrogen.

Summary. Molybdenum, iron and cal-

cium ions have been shown to be specifically required when *Azotobacter vinelandii* O is fixing atmospheric nitrogen. Ammonium ion eliminates the need for molybdenum and calcium and has a sparing action on the requirement for iron. High levels of iron stimulate the knallgas reaction of *A. vinelandii* O fixing N_2 in the presence of H_2, but molybdenum in the range tested had no effect on this reaction. Low concentrations of calcium ions in a nitrogen-fixing culture result in an extended lag phase of growth. Decrease in the concentration of phosphate in the presence of low levels of calcium brings about a further increase in the lag, but this increase is not observed if the calcium supply is adequate.

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Received September 21, 1956. P.S.E.B.M., 1956, v93.

Uricolysis in Normal and Leukemic Individuals. (22821)

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Hyperuricemia occurs with moderate frequency in leukemia and other blood dyscrasias. The hyperuricemia has been ascribed to increased nucleo-protein catabolism. In studies on the uric acid pool, Benedict(1) and her associates consistently found more urate produced than excreted. Using larger amounts of labeled uric acid, Wyngaarden (2) demonstrated that uricolysis occurs in significant amounts in humans. We attempted (3) to demonstrate the site of this uricolysis. The rate of urate disappearing from the formed elements of blood incubated with plasma was of a significant amount and could account for a large part of the degraded urate. Among our non-gouty hospitalized controls were several leukemics. These patients demonstrated a markedly lower rate of uricolysis than did the normals or even the gouty patients, the gouty patients being considerably lower than the normals. It is quite possible therefore, that elevated plasma urate levels in leukemics may be secondary, at least in part, to an impaired mechanism for uricolysis.

Methods. 50 ml of heparinized blood were obtained. A 5 ml sample was removed to serve as a whole blood specimen and the rest centrifuged. The plasma was removed and the buffy coat pipetted off. The buffy coat was centrifuged in a narrow tube in either the patient's own plasma or isotonic saline. After 3-4 separations, high concentrations of white blood cells relatively free of red blood cells were obtained. The red blood cell preparations were obtained by pipetting the red cells from the bottom of the tube and freed of white blood cells in the same manner as above. Each patient had the following samples incubated at 37° under 5% CO_2–95% O_2: 1. Whole blood. 2. Plasma. 3.White blood cells (0.5 ml) and the same patient's plasma (3. ml). 4. Red blood cells (0.5 ml) and the same patient's plasma (3. ml). Blood counts were done on preparations 3 and 4. On white cell preparations, the red counts

TABLE I. Mean Percentage Decrease in Plasma Urate after Incubation.

Subjects	No. of determinations	Mean plasma level	Whole blood	Plasma	White cells	Red cells
Normals	25	5.1 ± .1*	34.4 ± 2.3	2.3 ± 2.1	39.0 ± 2.2	29.8 ± 2.5
Leukemics	11	9.5 ± .2	22.4 ± 1.0	4.2 ± .3	21.4 ± .7	13.0 ± .7

* ± stand. error.

were less than 0.5 million, and in the red cell preparation, the white counts were less than 1000. For technical reasons, the patients chosen had increased leukocyte counts ranging from 18,000 to 542,000. Samples of plasma were withdrawn at 0, 4 and 24 hours of incubation and the plasma urate determined by the Yü modification of the Buchanan, Block and Christman method.

The results of 25 determinations on normal hospitalized patients as compared with 11 determinations on 7 leukemic patients are given in Table I.

To prove that the diminished rate of uricolysis was not related to the serum urate level, samples of normal sera were fortified by the addition of urate to hyperuricemic levels with no significant change in the rate of uricolysis. Of the leukemic patients, 3 had myelogenous leukemia, and 4 had lymphatic leukemia. There was no significant difference between the two types of leukemias we studied so far as diminished rates of uricolysis nor could differentiation be made as to the type of leukemia from the pattern of uricolysis. Aside from uricase, which has never been demonstrated in humans, 2 known enzyme systems are capable of oxidizing urate at a physiological pH. The first of these is peroxidase, which will oxidize urate in the presence of peroxide or a source of peroxide (as glucose and glucose oxidase) and the second is cytochrome-cytochrome oxidase(4). The end products of the peroxidase degradation were studied by Agner and by Tuttle and Cohen. The end products of cytochrome-cytochrome oxidase degradation have not been extensively studied.

Peroxidase is present to 1-2% of the dry weight of leukocytes of the myeloid series, while erythrocytes have both cytochrome and peroxidative activity(5). Agner states that leukocytes isolated from the blood in myeloid leukemia were very rich in peroxidase. The enzymatic activity of leukocytes in infections and in chronic myelogenous leukemia has been studied in regard to phosphatase content by Valentine(6). During a leukocytosis, the phosphatase activity may be as high as 5 times the normal and was found to be lower than normal in chronic myelogenous leukemia. Variable amounts of other enzymes are therefore also probable in disease states.

All urate determinations were total chromogen determinations and were not subjected to uricase degradation. Serious errors may still arise using a specific enzyme like uricase because of oxidation of other chromogenic substances or binding by the buffer of still other chromogenic substances(7). It is conceivable that uricolysis may proceed at a normal or even accelerated rate in leukemias but that substances reducing arsenophosphotungstic acid may be liberated into the plasma with the result that a defect in uricolysis is being demonstrated in the sample taken.

Summary. 1. Uricolysis occurs in whole blood as well as in plasma incubated with white or red blood cells. The rate of uricolysis is not dependent on the hyperuricemia in normals. There is marked defect in uricolysis in leukemics under the same experimental conditions. 2. Hyperuricemia in leukemia, may be in part due to diminution in the rate of uricolysis.

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Received September 26, 1956. P.S.E.B.M., 1956, v93.

Antiglobulin Test in Anemia Caused by Acetylphenylhydrazine. (22822)

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Following intravenous injection of red cells from one individual to another of the same species, certain sensitizing antibodies may develop. They may be detected *in vitro* by the antiglobulin test (Coombs Test). This test depends upon the sensitization of red cells by such antibodies, with subsequent agglutination of these sensitized cells by antiglobulin serum. At times, autosensitization may occur, as in certain types of acquired hemolytic anemia. It has generally been concluded that, for unknown reasons, the affected individual developed antibodies to his own red cells, which were then destroyed, thus accounting for the anemia. On the other hand, it is possible that a positive Coombs test may be caused by injury of some other type, and may not involve a specific immunological reaction. This was suggested by a report of Muirhead, Groves and Bryan(1) who found a positive antiglobulin reaction in dogs during anemia produced by acetylphenylhydrazine. We have repeated the experiments, but failed, however, to confirm them. A preliminary search to account for the difference in results was unsuccessful so the disparity remains unexplained. Since these findings are of considerable theoretical importance, we feel the data should be recorded.

Methods. The technic of Muirhead, Groves and Bryan(1) was used, and, in addition, certain modifications were introduced in attempts to account for differences in results. Two different types of Coombs serum were prepared. The first (Serum A) was made by injecting whole canine serum into rabbits, exactly as directed(1). Since it seemed possible that the positive reactions reported(1) were due to antibodies other than antiglobulins, a second type of Coombs serum (Serum B) was prepared as follows: the globulin fraction was separated from canine plasma by ammonium sulfate precipitation. An alum precipitate was prepared from this(2). Rabbit antiserum was then produced and absorbed against pooled canine erythrocytes. The titers of the 2 types of Coombs sera were nearly the same, with identical specificities. On each day, both sera were used, and each was controlled by an indirect Coombs test against the test cells sensitized by a canine isoantibody. The dogs were given a single intravenous dose of 40 mg/kg of acetylphenylhydrazine as directed(1). Direct Coombs tests were performed at frequent intervals thereafter. In addition, in each instance, 2-fold serial dilutions of the Coombs sera were made in an attempt to demonstrate the prozone phenomenon reported(1). In doing these titrations some of the tests were set up using 1 ml of a 3% suspension of test erythrocytes rather than the 0.5 ml of a 2% suspension as was previously reported(1). Dogs 1-4 were done with the former, and dogs 5-8 with the latter. On several occasions both methods were used together and no difference was detected. The criteria for a positive reaction were those used by Muirhead, Groves and Bryan(1). Hematocrit determinations were done in Wintrobe tubes using heparinized venous blood.

Results. The results are summarized in the Table. Since all titrations were negative, only the result of the ordinary direct Coombs test are given. With the exception of one weakly positive test and 2 doubtful tests on the 9th day in dogs 1, 2 and 3, all were clearly negative. It may be of significance that these positive tests were all obtained against the Coombs serum produced against the whole canine serum. The tests using the true antiglobulin serum were uniformly negative. Whether the conflicting results of our experiments and those of Muirhead *et al.* were due to difference in the antiglobulin sera used remains undetermined. Further work along these and similar lines seems necessary.

Summary. No evidence of a positive antiglobulin test was found in dogs following injection of acetylphenylhydrazine. This is in

TABLE I. Reactions of Canine Erythrocytes with 2 Types of Coombs Sera on Representative Days following a Single Intravenous Dose of Acetylphenylhydrazine.

Dog #	1			2			3			4		
Day after phenyl-hydrazine	Hct, %	Serum A*	Serum B†	Hct, %	Serum A	Serum B	Hct, %	Serum A	Serum B	Hct, %	Serum A	Serum B
0	49	—		45	—	—	56	—	—	51	—	—
1	41	—	—	34	—	—	51	—	—	44	—	—
4	31	—	—	26	—	—	40	—	—	36	—	—
7	27	—	—	28	—	—	27	—	—	28	—	—
9	30	±	—	31	±	—	34	1+	—	30	—	—
11–14	39	—	—	37	—	—	42	—	—	38	—	—
15–18	42	—	—	37	—	—	43	—	—	41	—	—
19–24	49	—	—	47	—	—	51	—	—	46	—	—

Dog #	5			6			7			8		
Day after phenyl-hydrazine	Hct, %	Serum A*	Serum B†	Hct, %	Serum A	Serum B	Hct, %	Serum A	Serum B	Hct, %		Serum B
0	46	—	—	41	—	—	53	—	—	44		—
1	37	—	—	36	—	—	42	—	—	23		—
4	33	—	—	23	—	—	31	—	—	29		—
7	31	—	—	30	—	—	27	—	—	26		—
9	33	—	—	30	—	—	27	—	—	25		—
11–14	36	—	—	33	—	—	35	—	—	34		—
15–18	38	—	—	35	—	—	39	—	—	34		—
19–24	46	—	—	46	—	—	44	—	—	42		—

* Serum A: Antiserum against whole dog serum.
† Serum B: " " dog globulin (see text).

contradiction to the results of others.

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Received October 4, 1956. P.S.E.B.M., 1956, v93.

Effect of Russell's Viper Venom (Stypven) on Stuart Clotting Defect. (22823)

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In a study of patient (R.S.) with congenital hemorrhagic disorder of moderate severity, Lewis, *et al.*(1) showed he had a prolonged one-stage prothrombin time which was corrected by normal plasma or serum but not by barium sulphate-adsorbed plasma or serum. The 2-stage prothrombin was normal and prothrombin consumption abnormal. These authors believed that the factor deficient in this patient was identical to that previously described by Alexander, *et al.*(2) Koller, *et al.*(3) and Owren(4) and now referred to as SPCA, factor VII or proconvertin (the 3 terms being used synonymously). It has now been established that factor VII (SPCA or proconvertin) is not essential for generation of blood thromboplastin(5,6). In a re-study of patient (R.S.) it has been found that the patient's serum is deficient in a factor essential for blood thromboplastin formation(7). Thus if various concentrations of normal serum in the patient's serum are prepared and incubated with normal $Al(OH)_3$-adsorbed plasma and platelets in the thromboplastin generation test, maximum yield of thromboplastin generated

is proportional to amount of normal serum in the mixture. The factor deficient in this patient therefore differs from factor VII. However the two factors appear to have many properties in common, both being relatively heat stable, present in serum as well as plasma and necessary for a normal one-stage prothrombin time(7).

Since Russell's viper venom (Stypven) completely corrects the prolonged one-stage prothrombin time of plasma deficient in factor VII(8,9) it appeared reasonable to determine whether Stypven would also correct the clotting defect of the patient (R.S.)

Materials and methods. Whole blood was collected in glass tubes, and allowed to clot and stand at 28°C for 24 hours before separating the serum. Plasma was obtained by adding 9 parts whole blood to 1 part 3.8% trisodium citrate and centrifuging at 3,000 rpm. Russell's viper venom (Stypven), obtained from Burroughs Wellcome and Co., was diluted with distilled water. Platelet suspensions were prepared from a normal subject by the technic used in the thromboplastin generation test(10). A 0.1% suspension of crude cephalin(11) in distilled water was used. Modified one-stage prothrombin times were determined by mixing 0.1 ml 0.1% cephalin suspension, 0.1 ml platelet suspension or fresh thromboplastin solution (Difco), 0.1 ml Stypven solution, 0.1 ml plasma and 0.1 ml 0.025M $CaCl_2$ in the order named.

Results. Effect of varying concentrations of Stypven on modified one-stage prothrombin time of patient's plasma. Various concentrations of Stypven were prepared ranging from 1 in 1,000 to 1 in 100,000 and the modified one-stage prothrombin time of normal or patient's (R.S.) plasma determined using Difco brain thromboplastin and each dilution of Stypven (Table I). It is to be noted that varying the concentration of Stypven did not result in any significant correction of the patient's prothrombin time, which remained between 2½ and 3 times that of normal control for the particular dilution of Stypven under test. Similar results were obtained if either a platelet suspension or a 0.1% suspension of crude cephalin was substituted for the brain thromboplastin used in the above experiment.

TABLE I. Effect of Concentration of Stypven on Modified One-Stage Prothrombin Time Using Difco Rabbit Brain Thromboplastin.

.1 ml plasma, .1 ml Difco brain thromboplastin, .1 ml of Stypven in conc. listed below, .1 ml 0.025M $CaCl_2$,	Clotting times, sec.	
	Normal plasma	Patient's plasma
1 in 1,000	8.9	23.5
2,500	5.5	15
5,000	5	14.6
10,000	6.6	18.8
20,000	8.5	22
50,000	11	27
100,000	11.8	35
Saline control	13.8	42

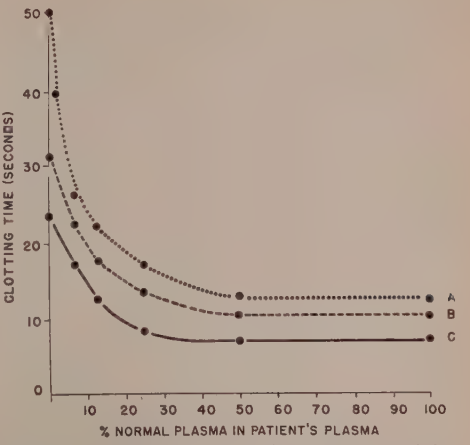

FIG. 1. Dilution curves obtained by determining one-stage "prothrombin" times of various dilutions of normal plasma in patient's (R.S.) plasma using, as sources of thromboplastin, a mixture of brain thromboplastin and (A) saline, (B) "Stypven" diluted 1 in 60,000 and (C) "Stypven" diluted 1 in 10,000.

Effect of diluting patient's plasma with normal plasma. Serial dilutions of patient's plasma in normal plasma were prepared and the modified one-stage prothrombin times of each dilution determined using Difco brain thromboplastin with either Stypven or saline (Fig. 1). Two concentrations of Stypven were used, 1 in 10,000 which was found to be the optimal dilution for the batch of Stypven used in this experiment and a 1 in 60,000 dilution. The result of such an experiment is shown in Fig. 1, where it can be seen that the

3 curves are roughly parallel: the one-stage prothrombin time and modified one-stage prothrombin, increasing in the same proportion when the concentration of normal plasma in the patient's plasma is diminished.

Discussion. It has already been shown that the patient (R.S.) is not deficient in either PTC or prothrombin and that mixtures of plasma from a case of congenital deficiency of SPCA (factor VII) and the patient's plasma are mutually corrective(7). The factor deficient in the patient differs from factor X since this factor affects the yield but not the rate of blood thromboplastin formation. The factor, deficient in the patient, also differs from AHF and factor V which are not present in normal serum and from the Hageman factor(12), PTA(13) and the fourth thromboplastin factor(14) since deficiencies of these factors do not affect the one-stage prothrombin time.

Telper, *et al.*(15) have recently described a patient with a congenital hemorrhagic diathesis due to deficiency of a factor which resembles factor VII but is necessary for blood thromboplastin formation. This factor, which is referred to as the Prower factor, resembles that deficient in our patient. The clotting defect is, however, completely corrected by Stypven while the present results show clearly that the clotting defect in the patient (R.S.) is not corrected by Stypven. This difference cannot be explained on a technical basis as one of the technics used in the present work was the same as that employed by Telper, *et al.*, and similar results were obtained using modifications of this technic. Moreover the difference is not merely a quantitative one for the patient of Telper, *et al.* had a one-stage prothrombin time which was more than twice the normal control indicating that the clotting deficiency was a moderately severe one. The two clotting defects must therefore be distinct and the factor deficient in the patient (R.S.) is referred to as the Stuart factor after the patient's surname.

The possibility that the patient (R.S.) is deficient in two factors has to be considered. A multiple congenital defect would imply that the patient was deficient in the Stuart factor and one or another *new* clotting factor including possibly the Prower factor since a deficiency of all the other known coagulation factors including Christmas factor (PTC) has been excluded. Since 2 other unrelated patients suffering from congenital hemorrhagic diatheses have been studied and found to have identical clotting defects(1,7), the multiple deficiency hypothesis is highly unlikely. The probabilities of the same double homozygosity or homozygosity at one locus, and heterozygosity at the other occurring independently 3 times in a small population are exceedingly small. The possibility of a multiple congenital deficiency can therefore be discarded with a reasonable degree of safety.

The finding that Stypven does not correct the Stuart defect however, does more than establish its separate identity. It is of practical importance since a simple prothrombin assay method has been described in which it is assumed that the one-stage "prothrombin time" is a true measure of prothrombin when Stypven is added to the brain thromboplastin (16). Such an assay procedure however is a function of both prothrombin and Stuart factor. It has been pointed out elsewhere(7) that the usual assay procedures for factor VII (proconvertin) as described by Owren(17) and Koller *et al.*(3) measure both Stuart factor and factor VII. These asbestos filtered plasmas retain considerable prothrombin but are deficient not only in factor VII (proconvertin) as these workers have assumed but Stuart factor and probably Prower factor. However, the simple addition of Stypven to such substrate plasmas should allow these assay technics to measure specifically the Stuart factor.*

Summary. It is shown that Russell's viper venom (Stypven) does not correct the prolonged one-stage prothrombin time of patient with a congenital hemorrhagic diathesis previously thought to be deficient in factor VII. The factor deficient in this patient which is referred to as the Stuart factor, is readily distinguishable from both the Prower factor and factor VII since the prolonged prothrombin times of plasma deficient in either of these

* To be published.

two factors are completely corrected by Stypven. The finding that Stypven does not correct the clotting defect of Stuart factor deficient plasma implies that certain modified one-stage "specific" assay methods for prothrombin using Stypven as a source of factor VII are not, in fact, specific, and measure both prothrombin and Stuart factor.

The author wishes to thank Dr. John B. Graham for his helpful suggestions and interest.

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Received October 8, 1956. P.S.E.B.M., 1956, v93.

Oxygen Uptake and Lactate Formation of HeLa Cells.*† (22824)

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HeLa cells were obtained from an epidermoid carcinoma of the cervix and started as a tissue culture in 1951(5). After 4 years *in vitro*, smears stained by Papanicolou's technic (4) indicated that HeLa had malignant features. It has been grown by Toolan in cortisone-treated rats and in human subjects in terminal stages of another neoplastic process (5). The cells are uniform in appearance as compared to mixed cell types found in tissue. They can be easily separated without apparent injury. Cell number or weight can be determined quite accurately since there is little interstitial material. For these reasons HeLa makes an ideal cell suspension on which to study metabolism. Our primary objective was to compare metabolism of human HeLa cells to that reported characteristic of animal tumors(2). Originally Gey started HeLa in medium which contained human serum. Other laboratories§ adapted a line of HeLa cells to grow in medium containing horse serum. Our second objective was to compare metabolism of cells cultured in human serum‖ with that of cells cultured in horse serum.

Procedure. At start of this study 2 lines of HeLa were carried in medium containing 40% Earle solution, 20% chick embryo extract (50:50) and 40% horse or human serum. It was desired to carry one line of cells

* Supported by grant from Ladies Auxiliary, Veterans of Foreign Wars, Dept. of Nebr.

† Preliminary report presented Physiol. Meet., Rochester, 1956.

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§ Original cultures obtained from Dr. W. R. Earle.

‖ Serum furnished by Amer. Red Cross (Dr. R. M. Joyer) and Corn State Laboratories (Dr. N. K. Jarvis).

in human medium. Subcultures of each line were placed in semi-synthetic medium which had proven adequate for indefinite growth of Earle's Strain L cells. An initial solution contained 0.25 g yeast¶ and 0.25 g lactalbumin hydrolysates/liter Earle solution. Horse or human serum (10%) was added before use. Culture medium was changed 3 times weekly and all metabolic determinations made the day following a culture change. Determinations on HeLa were made in 2 suspending solutions. In one, phosphate solution alone was used, in the other 10% neutralized horse or human serum(10) was added to it. Respiration was determined by direct method of Warburg(9) which does not allow use of Earle solution because of its high bicarbonate content. Phosphate buffered solution (pH 7.4) adopted for use, contained the following materials per liter: 8.12 g. NaCl, 0.28 KCl, 0.15 $CaCl_2$, 0.33 $MgSO_4 \cdot 7\ H_2O$, 0.28 Na_2HPO_4, 0.05 $NaH_2PO_4 \cdot H_2O$, 1 Glucose, 0.25 lactalbumin hydrolysate, 0.25 yeast hydrolysate. Lactate was determined chemically by the method of Barker and Summerson(1). Concentration in one reaction vessel was obtained immediately after equilibration for temperature and in another an hour later. Difference between concentrations in the 2 vessels was taken as lactate formation. During experimentation, number of HeLa cells in a reaction vessel was determined by counting on a hemacytometer. To obtain dry weight intercellular material was digested with 0.25% Difco trypsin (1:250) in Earle solution. The cells were collected by centrifugation, resuspended in Earle solution, counted, then collected again and dried at 110°C to constant weight. Three determinations indicated that one million HeLa cells weighed 0.99 mg. Phenylenediamine was used to obtain a measure of functional cytochrome reserve. This was done by adding p-phenylenediamine from side arm of reaction vessel so that final concentration was 0.02 M. Respiration was then compared with that in control flask without p-phenylenediamine. In preliminary respiration experiments, 2 methods of separating cells were tried. Trypsin was used in one method. In the other, cells were brushed from floor of T-60 flasks with brush made of stainless steel and nylon bristles and separated further by filling and discharging them through a pipette 15 times. Since trypsin appeared to depress respiration in some cases (Table I), use of nylon brush was considered the better method for cell separation. It is necessary to assume a constant rate of oxygen uptake for a definite interval to express Q_{O_2} values as mm^3/hr. Rate of oxygen uptake for practical purposes was constant for one hour, but slightly less at 2 hours (Fig. 1). Subsequent determinations were run for exactly one hour. Respiration was higher at 38°C than at 37.5°C ($P<.01$) but the lower temperature was used for metabolic determinations because incubators used for growing cultures had been maintained at 37.5°C.

¶ Nutritional Biochemicals Corp.

TABLE I. Effect of Separation Methods on Respiration of HeLa.

Method	Culture serum	Warburg sol.	Temp., °C	Q_{O_2}	P
Brush	Horse	Saline	37.5	2.2 (8)	
Trypsin			37.5	2.2 (5)	
Brush	Horse	Serum	38.0	7.7 (2)	>.05
Trypsin			38.0	5.8(13)	
Brush	Human	Saline	37.5	8.4 (6)	<.05
Trypsin			37.5	4.7 (5)	

Figures in parentheses indicate No. of determinations.

Results. Comparison was made either between quotients obtained in saline or between those in saline-serum. Without exception, all metabolic quotients were higher for HeLa cells grown in medium containing human se-

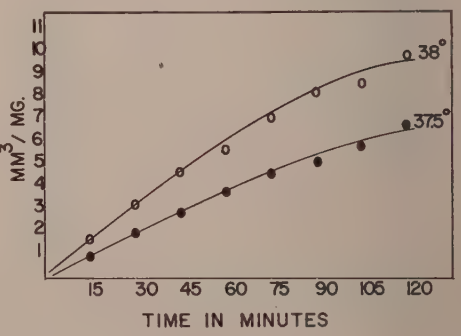

FIG. 1. Oxygen uptake of HeLa in neutralized horse serum.

TABLE II. Respiration and Glycolysis at 37.5°C of HeLa Grown in Media Containing Either Human or Horse Serum.

Culture serum	Warburg solution	Q_{O_2}	$Q_{LA}^{O_2}$	$Q_{LA}^{N_2}$	P-PD
Horse	Saline	2.2 (13)	6.7 (3)	3.2 (2)	11.9 (3)
Human	"	6.3 (11)	10.2 (12)	8.4 (6)	14.3 (5)
Horse	" + horse serum	4.0 (13)	7.2 (4)	5.2 (5)	21.7 (4)
Human	" + human "	6.0 (4)	12.0 (4)	5.5 (5)	26.3 (4)

All values expressed as mm^3/mg dry wt/hr. Figures in parentheses indicate No. of determinations. P-PD = Q_{O_2} when 0.02 M p-phenylenediamine added.

rum than those grown in a horse serum medium (Table II).

Quotients determined in saline and in serum were about the same order of magnitude. There were exceptions. Respiration of HeLa adapted to grow in horse serum, when determined in saline, was lower than similar determinations in horse serum ($P<.05$). Phenylenediamine stimulated respiration of cells in solution containing serum to a greater extent than it did when saline alone was used; this was true for cultures which had been grown in horse serum ($P<.01$) or human serum ($P<.05$).

It has been pointed out by Warburg, Burk and others(2,9) that tumors have a metabolism that differs from normal tissues. HeLa cells, carried by tissue culture technic, metabolized in some ways like *in vivo* derived tumor slices but in other ways like normal tissue(6) (Table II). Metabolism of HeLa cells was similar to that reported for tumors, in that respiration (Q_{O_2}) was moderately low and aerobic lactic acid formation ($Q_{LA}^{O_2}$) moderately high. They exhibit metabolism dissimilar to that reported for tumors in that anerobic lactic acid formation ($Q_{LA}^{N_2}$) was low and added p-phenylenediamine stimulated respiration greatly (338%). The latter observation was indirect evidence that adequate functional cytochrome reserve was present. There was no Pasteur Effect ($Q_{LA}^{N_2} - Q_{LA}^{O_2}$) because anerobic glycolysis was not in excess of aerobic glycolysis. Respiration and anerobic glycolysis were about the same magnitude and thus Fermentation Excess ($Q_{LA}^{N_2} - 2Q_{O_2}$) was negative.

Discussion. Numerous workers have shown that animal cells grown in chemically defined medium need, among other things, certain amino acids and vitamins. In an attempt to obtain a simple homologous medium enzymatic lactalbumin hydrolysate was used because Melnick and Riordon(8) had found it to be growth stimulating; enzymatic yeast hydrolysate was used so that most B vitamins as well as amino acids would be present. Hydrolysates were added to the medium in concentration that would yield an amino acid solution very similar to that found in serum (7). The medium was sterilized by Selas filtration rather than by autoclaving(8) so that thiamin, folic acid and vit. B_{12} would not be destroyed. Preliminary results indicated that hydrolysate culture medium, as used in this study, compared favorably to Eagle's synthetic medium(3) supplemented with 10% serum.

One early observation which we did not readily accept was that aerobic glycolysis was higher than anerobic glycolysis even when bound bicarbonate was present in neutralized serum. Our repeat experiments gave similar results. Warburg(9) has shown that glycolysis of more than 20 different tumors was higher under anerobic than aerobic conditions. It is interesting to note that Hellerman collaborating with Gey(5) found that aerobic lactate was higher than anerobic lactate for the 14P rat fibroblast cultured *in vitro*.

Summary. Gey's strain HeLa cells were cultured in Earle T-60 flasks for almost a year in media composed of 0.25 g. yeast and 0.25 g. lactalbumin hydrolysate/l Earle solution with 10% human or horse serum. Respiration was determined manometrically and lactate formation chemically. In preliminary experiments, 0.25% Difco trypsin (1:250) when used to separate cells, depressed respiration in some instances. Respiration of sus-

pensions of HeLa was constant for an hour but decreased slightly after first hour. Metabolism of two lines of HeLa cells was compared. Respiration and glycolysis of cells grown in media containing homologous human serum was higher than that of cells grown in media containing horse serum. Human HeLa cells had a metabolism like that reported for tumor tissue in that respiration was low ($Q_{O_2} = 6.0$) and aerobic glycolysis moderate ($Q_{LA}^{O_2} = 12.0$). They differed from tumor tissue in that anerobic glycolysis was low ($Q_{LA}^{N_2} = 5.5$) and p-phenylenediamine stimulated respiration 338% ($Q_{O_2} = 26.3$). There was no Pasteur Effect and Fermentation Excess was negative.

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Received October 10, 1956. P.S.E.B.M., 1956, v93.

Absence of Appreciable Resistance to Growth Inhibiting Action of Cortisone After Prolonged Treatment.* (22825)

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Chronic administration of nitrogen retaining hormones, to the plateaued adult Norway rat, leads to period of rapid growth followed by attainment of a new weight plateau on which there is no further growth although dosage of hormone per animal or per unit weight, is unchanged. This phenomenon of plateauing on chronic treatment with anabolic hormones appears to be identical in protein anabolic steroids(1,2) in anterior pituitary growth hormone(3-5); and in anabolic steroids plus growth hormone(6). Glucocorticoids have been shown to have a striking growth inhibiting action(7,8) due to their protein catabolic action. It would, therefore, be of interest to determine if there is a failure of constant dosage of glucocorticoid to give a constant inhibition of growth; indeed, the data of Tolksdorf *et al.*(8) suggest that this might be the case.

The present investigation was performed to further clarify possible development of resistance to growth inhibiting action of glucocorticoids. Since the time required for growth cessation, on a constant dosage of anterior pituitary growth hormone, is highly sensitive to the dose level(9), a range of dose levels was employed.

Methods. A microcrystalline suspension of cortisone acetate† (Δ^4-Pregnene-17β, 21-diol-3,11,20-trione-21-acetate) in isotonic saline was prepared by trituration with Tween-80 (polyoxyethylene sorbitan monooleate). Concentration of the suspension was adjusted to contain 3.2 mg cortisone/ml and less than 0.2 % Tween-80. After dilution, the suspension was sealed in injection vials and stored at 2 to 4°C until just before injection. Single daily subcutaneous injections were given under strictly aseptic conditions, at systematically varied sites along dorsal surface of the rat. Dosage was adjusted every 2 days

* This investigation was supported in part by Research Grant No. 13, from Interim Research Committee, of Univ. of Alabama Medical Center.

†Cortisone used was generously contributed by Dr. C. J. O'Donovan and Department of Clinical Investigation, The Upjohn Co., Kalamazoo, Mich.

TABLE I. Weight Gain of Cortisone Treated Young Female Rats, in Successive 10 Day Intervals, before and during Treatment.

Cortisone dose, mg/kg/day	No. of rats	Wt gain (g) 10 days pretreat.	10 day intervals of treatment 1st	2nd	3rd	4th	5th	Mean wt (g) 1st day of treatment
0	15	40.9 ± 2.0*	35.3 ± 1.7	27.0 ± 1.6	19.2 ± 1.3	12.1 ± 0.9	12.3 ± 1.1	98.4
8	8	38.3 ± 2.8	17.0 ± 2.7	17.0 ± 2.3	14.5 ± 2.1	6.7 ± 1.8	5.2 ± 1.4	99.0
16	8	40.6 ± 2.6	5.7 ± 2.2	12.1 ± 2.8	10.8 ± 1.0	6.8 ± 1.7	6.2 ± 1.5	98.5
24	8	41.6 ± 3.1	−2.6 ± 2.8	8.9 ± 1.4	10.6 ± 1.6	3.1 ± 1.6	2.2 ± 2.2	99.6
32	8	41.9 ± 2.4	−4.1 ± 2.0	4.4 ± 2.1	6.1 ± 2.0	8.0 ± 2.1	3.1 ± 1.1	99.9

* Mean ± stand. error of mean in all cases.

to maintain dosage/kg body wt constant. The rats were rewarded with 0.5 ml imitation maple syrup after each injection. Split litter controls were given the same amount of syrup, but were not given injections of the suspending medium since previous work in this laboratory has shown 1 cc/day of 0.2% Tween-80 to be devoid of effect on growth. In experiments reported on anabolic hormones, stanolone (Androstane-17β-ol-3-one) was suspended in saline with Tween-80 in the same manner as cortisone and adjusted to concentration of 10 mg stanolone/ml. Ant. pituitary growth hormone was prepared in this laboratory by modification of the method of Wilhelmi, Fishman and Russell(10). The growth hormone was dissolved in sodium carbonate-saline at pH 9.5; adjusted to growth hormone concentration of 2 mg/ml; divided into injection vials and stored frozen at −30°C until just prior to injection. All experiments were carried out in the room in which rats had been raised from birth. Temperature was maintained at 22 to 24°C and all groups were fed Purina Laboratory Chow *ad lib*. The rats were an all black strain, developed in this laboratory from original Long-Evans stock. Rats on the cortisone experiment were young females with mean age of 38 days, and mean wt of approximately 100 g on the day treatment was initiated. Rats on the anabolic hormone experiments were plateaued young adult females, 8-10 months of age, having a mean wt of 250 g at onset of treatment.

Results. Table I gives growth of young female rats on cortisone treatment during 10 days prior to treatment and during 5 successive 10 day periods of treatment. The decrease in growth rate of control animals during each successive 10 day interval is the characteristic plateauing as adult size is approached. This is probably also the explanation of the successive decrease in growth in the group treated with 8 mg/kg/day. A complete cessation of growth was achieved only at the 24 mg/kg/day and 32 mg/kg/day dose levels, and then only during the first 10 day period. There appears to be no meaningful increase in growth in any group after the second 10 day period on treatment. Furthermore, during the final 10 day period, neither the 24 mg/kg/day, nor the 32 mg/kg/day group achieved a growth equal to 10% of that shown by normal controls when at the same body wt.

In comparison, Table II shows the typical decrease in resumed growth of adult animals given growth promoting hormones over intervals corresponding to those used in the cortisone experiments.

Discussion. We interpret the data presented above to indicate that no appreciable loss in growth inhibiting action of glucocorticoids occurs, at any dose level utilized, within a time interval comparable to that required for cessation of the resumed growth of adult rats treated with growth promoting hormones.

Summary. Cortisone acetate was given daily at 4 dose levels, for 50 days, to young female Long-Evans rats, initially 38 days of age and weighing approximately 100 g each. The upper 2 dose levels were in the range giving nearly complete cessation of growth. In the upper 2 dose levels, there appeared to be a slight reduction in growth inhibiting action after the first 10 days, but all 4 groups still had a marked depression of growth during the 40th to 50th day of treatment. For comparison, anterior pituitary growth hor-

TABLE II. Weight Gain of Plateaued Adult Female Rats Treated with Pituitary Growth Hormone and Stanolone, in Successive 10 Day Intervals before and during Treatment.

Treatment and dose	No. of rats	Wt gain (g) 10 days pretreat.	10 day intervals of treatment 1st	2nd	3rd	4th	5th
Growth hormone, 1 mg/rat/day	8	0.9 ± 2.0*	38.6 ± 2.8	21.8 ± 4.6	−5.8 ± 5.7	−3.0 ± 3.1	−1.6 ± 1.0
Growth hormone, controls	20	0.7 ± 0.7	4.9 ± 1.3	2.8 ± 1.1	−1.2 ± 1.3	2.9 ± 1.1	−2.4 ± 1.0
Stanolone, 5 mg/rat/day	6	1.2 ± 1.6	19.3 ± 2.3	6.3 ± 1.3	6.0 ± 0.5	3.7 ± 1.6	
Stanolone, controls	18	3.1 ± 0.8	2.3 ± 0.7	0.0 ± 1.4	5.1 ± 1.1	2.0 ± 1.2	

* Mean ± stand. error in all cases.

mone and stanolone were given to plateaued adult female rats of the same strain. These hormones, at dose levels capable of giving a major growth response during the first 20 days of treatment, gave little response after the first 20 days.

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Received October 11, 1956. P.S.E.B.M., 1956, v93.

Serum Protein-Bound Carbohydrates and Lipids in Experimental Tuberculosis. (22826)

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Guinea pigs inoculated with tubercle bacilli reveal characteristic serum protein changes which resemble to a great degree those in human tuberculosis; serum gamma globulin is elevated and albumin reduced(1,2). These serum protein changes, especially the gamma globulin elevation, have been related to chronic inflammation and hypersensitivity reactions. However, similar changes occur in chronic hepatic disease. It appeared therefore desirable to further characterize the serum protein changes produced by chronic tuberculosis.

A rise of serum mucoproteins (which are alpha globulins)(3) in experimental tuberculosis(4), in human tuberculosis(5) and in experimental brucellosis(6) has been demonstrated. This rise is absent in hepatic injury (7). Since methods have become available recently to demonstrate serum protein-bound lipids(8) and carbohydrates(9) by zone electrophoresis it appeared worthwhile to study their levels in experimental tuberculosis. To accentuate the serum protein changes, animals with long standing tuberculosis were investigated. The levels were compared not only with those of healthy control guinea pigs but also with animals in which treatment with

isoniazid temporarily prevented development of tuberculosis(10).

Materials and methods. Serum was obtained from 12 guinea pigs inoculated subcutaneously into the right inguinal region with 0.1 mg of tubercle bacilli, strain H37Rv, and exhibiting far advanced tuberculosis at autopsy. Another group of 7 animals similarly infected received 20 mg isoniazid daily for 2 months following inoculation and were free of tuberculosis grossly and microscopically at autopsy. All animals were sacrificed 8 to 16 months after inoculation simultaneously with 12 control guinea pigs kept for the same period and free of any tuberculosis at autopsy. The following were determined in the serum: total proteins by micro-Kjeldahl and Nesslerization; gamma globulin turbidimetrically according to Huerga and Popper (11); mucoproteins also turbidimetrically according to Huerga *et al.*(12); hexosamines according to Elson and Morgan(13); protein-bound hexoses according to Lustig and Langer(14) and total lipids turbidimetrically according to Huerga *et al.*(15). In addition 3 paper strips, on which serum had been placed, were exposed to vertical zone electrophoresis. One was subsequently stained with bromphenol blue for proteins(16), another with periodic acid leucofuchsin (Schiff reaction) for protein-bound carbohydrates(9) and a third one with Oil Red O, for protein-bound lipids according to Durrum(8). The relative amount of protein as well as protein-bound carbohydrates and lipids were determined by integrating photometric scanner (Analytrol). The amounts found were expressed in percentage, and absolute amounts were calculated using as reference for proteins the total protein; for protein-bound carbohydrates the sum of hexosamines and protein-bound hexoses; and for protein-bound lipids the total lipids. In addition lipid and carbohydrate content of each serum protein fraction was calculated and expressed in percent of that fraction.

Results. In guinea pigs with active tuberculosis serum albumin was moderately reduced and gamma globulin and mucoprotein were significantly elevated. These changes, as well as those to be reported below, were absent in inoculated animals in which isoniazid treatment prevented active tuberculosis.

Total serum lipids as well as lipoprotein fractions were not influenced by tuberculosis. Both total serum hexose and hexosamine were slightly elevated in active tuberculosis as were all protein-bound carbohydrates, if expressed in absolute amounts. However this increase was not statistically significant and the relative distribution of carbohydrates was not significantly altered. Carbohydrate concentration of gamma globulin however, was distinctly depressed in guinea pigs with tuberculosis while that of alpha globulin and beta globulin were not significantly altered.

The very small amount of carbohydrates in the large fraction of albumin discouraged calculation of this figure.

The failure of the carbohydrate moiety of the gamma globulin to rise parallel with protein, resulted in a lower carbohydrate concentration in this protein fraction. This reflected itself in the ratio of carbohydrate concentrations, (carbohydrate percent in alpha globulin/carbohydrate percent in gamma globulin) and in the alpha globulin/gamma globulin ratio of the proteins (Table I). The former increased, while the latter decreased in tuberculous animals.

There is an increase in mucoproteins in tuberculous animals.

Discussion. Since the most significant alteration in protein fractions in experimental guinea pig tuberculosis involves albumin and gamma globulin, it is not surprising that lipoproteins, mainly components of alpha and beta globulins, are not significantly altered in these conditions. The slight rise of alpha globulin under these circumstances is reflected in marked elevation of mucoproteins which are alpha globulins(3). Since mucoproteins represent only a very small fraction of alpha globulin their marked rise which also runs parallel with elevation of total carbohydrates, is not sufficient to produce significant changes of serum concentration of the alpha globulin. The relatively small rise is associated with a parallel rise of alpha globulin-bound carbohydrate which suggests that the excess amount of alpha globulin does not differ in carbohydrate content. However, this rela-

TABLE I. Serum Protein Fractions in Control and Tuberculous Guinea Pigs.

	Controls		Tuberculous active			Tuberculous successfully treated		
					OD			OD
	Mean	SEm	Mean	SEm	SE-diff.	Mean	SEm	SE-diff.
No. of animals	14		12			7		
Albumin, g %	3.1	.13	2.6	.03	3.6	3.1	.10	.0
α-globulin, "	1.6	.13	1.9	.04	2.1	1.7	.04	.7
β-globulin, "	.9	.04	.8	.05	1.5	.8	.06	.0
γ-globulin, "	.6	.05	1.6	.07	40.0	.8	.10	1.8
Mucoproteins, mg %	323	21	537	24	6.5	346	15	.9
Total lipids, "	217	6.2	201	16	.9	191	17	1.4
α-lipoproteins, "	128	7.0	131	17.7	.1	130	17	.1
β-lipoproteins, "	69	7.0	64	8.0	.5	57	6.4	1.2
Protein-bound hexose, "	110	9.4	119	5.0	.8	103	44	.7
Hexosamine, "	174	9.0	194	6.6	1.9	166	3.5	.8
Protein-bound CHO, "	296	12.3	312	8.6	1.1	269	6.3	1.2
Albumin-bound CHO, "	18	4.0	16	3.5	.4	13	3.5	.9
α-globulin-bound CHO, "	205	7.5	222	7.7	1.6	191	5.3	1.5
β-globulin-bound CHO, "	43	3.2	42	3.0	.2	35	2.0	2.2
γ-globulin-bound CHO, "	22	1.6	33	2.6	3.6	27	2.0	2.0
CHO, % in α-globulin	12.3	.6	11.3	1.0	.8	11.3	.8	1.0
" , " " β-globulin	4.6	.4	5.2	.3	1.2	4.4	.5	.3
" , " " γ-globulin	4.8	.6	2.3	.2	4.0	4.7	.3	.2
α-globulin/γ-globulin	3.5	.3	1.2	.1	20.0	2.5	.3	2.3
CHO, % in α-globulin / CHO, % in γ-globulin	3.4	.4	6.3	.9	9.4	2.9	.5	2.2

SEm is stand. error of the stand. dev.

$\frac{\text{OD}}{\text{SE-diff.}}$ is the observed difference between control and exp. group divided by stand. error of that difference (above 2.5 significant).

tively small increase prevents a more definite conclusion. Similarly the constancy of the beta globulin level and the small amounts of carbohydrate-bound albumin do not favor any calculation.

However, the contrast between the marked rise of serum gamma globulin with only a small elevation of its carbohydrate moiety, best reflected in the difference between the alpha globulin/gamma globulin ratio of protein and the ratio of the carbohydrate concentration of these proteins, permits the conclusion that the excess gamma globulin formed in tuberculosis is poor in carbohydrates. If calculations of the carbohydrate concentration of the excess gamma globulin in the tuberculous animals are permissible from the averages, this increment in the amount of 1 g per 100 ml has a carbohydrate moiety of almost 1% in contrast to the gamma globulin in control guinea pigs with a mean carbohydrate concentration of 4.8%. This is in contrast, for instance, to the elevated carbohydrate concentration of the excess gamma globulin in myeloma(17,18) and the unchanged concentration in infectious mononucleosis(19), experimental brucellosis(6) and hepatitis(20), and the small differences recently demonstrated by Müller-Eberhard and Kunkel(21) in the carbohydrate concentration in various components of gamma globulin in normal and abnormal circumstances. The findings in experimental tuberculosis suggest that the excess gamma globulin represents a reaction globulin(22) different in nature from the normal gamma globulin and possibly formed in other sites.

Summary. The previously demonstrated reduction of serum albumin and elevation of gamma globulin and mucoprotein in experimental guinea pig tuberculosis is associated with a slight elevation of total serum hexose

which is to the greatest extent accounted for by a slight rise of the carbohydrate containing alpha globulin. No evidence was obtained that the carbohydrate content of this small increment differs from that of the normal alpha globulin. In contrast the marked rise of gamma globulin is associated with an only very small increase of gamma globulin associated carbohydrates. This suggests that the excess gamma globulin in experimental tuberculosis differs from the normal gamma globulin by a very low carbohydrate content and suggests a different nature and possibly a different origin of this increment. The serum protein-bound lipids are not altered in experimental tuberculosis.

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Received October 15, 1956. P.S.E.B.M., 1956, v93.

Increased Metabolism in Fat-Deficiency: Relation to Dietary Fat.* (22827)

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Elevation of metabolic rate is consistently observed in rats fed a diet devoid of fat. As early as 1931, Wesson and Burr made reference to increased basal and carbohydrate assimilatory rates(1), findings which were confirmed and extended by Burr and Beber(2). It has been pointed out that disturbance in energy balance as indicated by increase in basal oxygen consumption is probably the earliest and most fundamental manifestation of the fat-deficiency syndrome, being recorded within 7-14 days after the start of the experimental diet in rats(3,4).

The manifestations of fat-deficiency are corrected by the administration of linoleic acid in amounts too small to exert an influence as a metabolite (*i.e.* "fat as fat *per se*"), indicating the specific role of this fatty acid as an essential substance(5). It is the purpose of this presentation to report the effect on oxygen consumption and body weight of

* Supported in part by grant from U.S.P.H.S.

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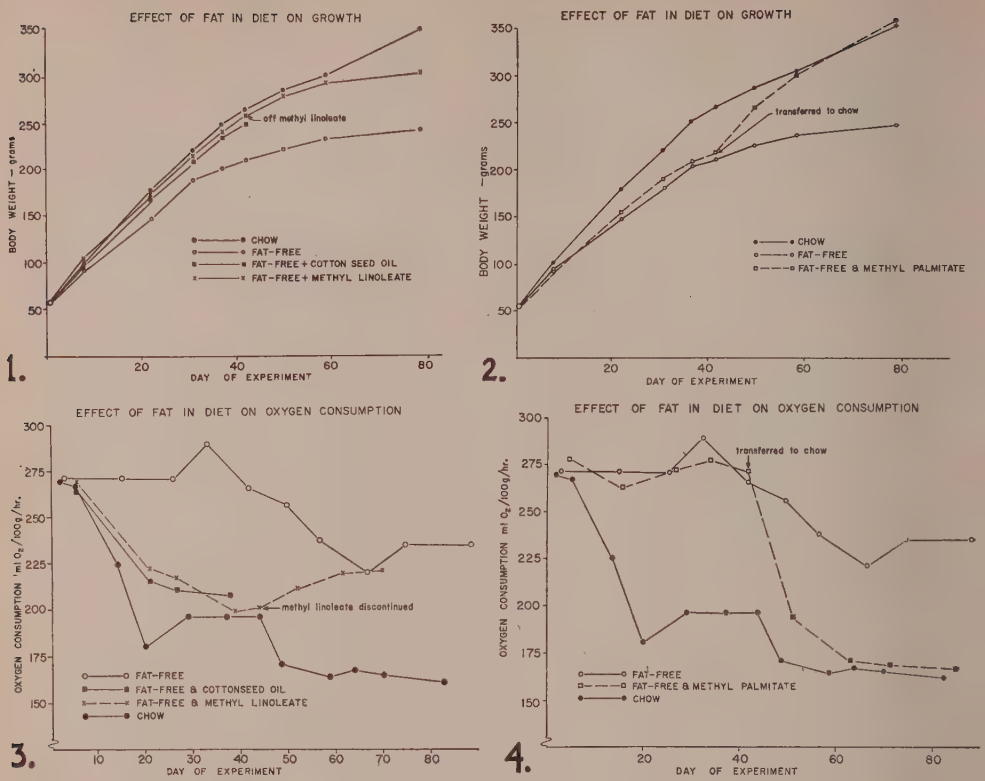

FIG. 1-4.

small amounts of saturated and unsaturated fatty acid preparations.

Materials and methods. Rats of the Holtzman strain were placed on experimental diets at weaning. Group I (12 rats) received the fat-free diet regularly used in this laboratory (3). Group II (12 animals) received the same fat-free diet supplemented by the daily administration for 6 weeks of 100 mg methyl linoleate‡ directly into the mouth via syringe. Tocopherol was added to the methyl linoleate for antioxidant effect in ratio of 1 to 100. It became necessary to discontinue this supplement after 6 weeks because of unexpected unavailability. Group III (12 rats) was fed the same fat-free diet similarly supplemented by daily administration of 100 mg methyl palmitate. Original plans for transfer from the palmitate to methyl linoleate were impossible, and the animals were transferred to laboratory chow.§ Group IV (12 rats) received the same fat-free diet similarly supplemented daily for 40 days with 200 mg cottonseed oil‖ which contains approximately 50% linoleic acid. Group V (15 rats) was fed laboratory chow. The animals were weighed weekly and observed for development of dermatitis. Oxygen consumption was determined weekly on 5 animals from each group according to a method previously described(3), modified by addition of a circulating pump. All determinations were carried out in an air-conditioned room with animals in a fasting, resting state; duplicate prolonged tracings were made after the animal had become motionless. The duration of the experiment was 11 weeks.

Results. The effects of various diets on growth are depicted in Fig. 1 and 2. It will

‡ Obtained from Hormel Institute, Austin, Minn.

§ Purina.

‖ Wesson.

be noted that supplementation with methyl linoleate resulted in gain entirely comparable to that achieved by animals fed laboratory chow, whereas an equal amount of saturated fatty acid (methyl palmitate) resulted in growth identical to that produced by feeding the fat-free diet alone. Following discontinuation of methyl linoleate, growth continued unaltered for approximately 20 days, after which it decelerated so that at 80 days, the mean weight of this group was significantly lower than that of chow-fed animals. In contrast, transfer of the methyl palmitate group to chow resulted in a pronounced acceleration in weight gain, which reached the level of chow-fed animals within a period of 20 days.

The basal oxygen consumption rates in the group receiving linoleate and cottonseed oil supplements were not significantly different from that of chow-fed animals (Fig. 3). Following withdrawal of methyl linoleate, oxygen consumption returned gradually to the level of fat-free controls. Comparison of these findings with those shown in Fig. 4 reveals that supplementation of fat-free diet with methyl palmitate produced no change in oxygen consumption rate. Following transfer to laboratory chow, a pronounced decrease was noted after 10 days and within 20 days the rate was identical with that of the chow-fed animals.

Although characteristic dermatitis developed in fat-free animals after approximately 9 weeks, no such changes were observed in any other groups. In the group in which supplementation with methyl linoleate was withdrawn after 6 weeks, no skin changes were observed during the remainder of the experiment although significant alterations in oxygen consumption and weight gain were observed.

Discussion. The amount of methyl linoleate administered in these experiments constituted the equivalent of approximately 1.5% of the total daily caloric intake as fat. It seems clear that the effects on oxygen consumption and body weight cannot be attributed to the simple caloric contribution of linoleate but to the provision of a specifically required substance. This conclusion is supported by the failure of a calorically equal amount of methyl palmitate to influence either oxygen consumption or weight. Although absorbability of tripalmitin from the gastrointestinal tract is known to be poor(6), that of palmitic acid appears to be great enough(7) to substantiate the use of methyl palmitate as an experimental control.

Supplementation with cottonseed oil to provide 3% of the calories as fat resulted in changes essentially identical to those following methyl linoleate alone, indicating that the effects were due to the linoleic acid content. Deuel(8) has shown that the increase in weight of male rats receiving 50 mg methyl linoleate daily approached that found in animals receiving 53% of the calories as cottonseed oil and has suggested that larger doses, 100 mg or more, might maintain weight comparable to rats receiving the high fat diet.

Recently, Hansen and coworkers(9) have reported that infants fed to satiety with skim milk (1% calories as fat and 0.04% as linoleic acid) consumed more calories per kg of weight gain than did infants ingesting a standard milk mixture providing 38% of the calories as fat and 0.9% as linoleic acid. When the skim milk diet was supplemented daily with an amount of methyl linoleate equivalent to 1% of the calories, the daily caloric consumption per kg body weight decreased 20-30% without affecting weight gain or activity. These findings suggest that fat-deprivation increases metabolism in human infants as in rats, with resultant increased caloric requirements and intake. The implications of these observations on principles of infant feeding are obvious, particularly in view of the popularity of low-fat milk mixtures in clinical practice.

Summary. Disturbance in energy metabolism, as indicated by increased basal oxygen consumption and growth retardation, is prevented when rats receiving a fat-free diet are given daily supplements of methyl linoleate (100 mg) or cottonseed oil (200 mg). However, similar supplementation with a saturated fatty acid (methyl palmitate, 100 mg) in no way modifies the development of the fat-deficiency syndrome.

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Received October 15, 1956. P.S.E.B.M., 1956, v93.

Improved Procedures for Preparation of Tissues for Chemical Analyses.* (22828)

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Many years of work on histochemical characterization of various tissues have required different technics for their preparation for precise chemical analyses. Previously, some soft tissues were minced and aliquots of wet minced tissue were weighed for all analyses: skeletal muscle(1,2), brain(3) and heart(4); some tissues were minced, dried and smashed in a special apparatus for all analyses: liver (5,6), skin(7,8), kidney(9), cartilage(10,11).

The method described here has many advantages: it is saving in time and effort while producing material excellent for sampling; it permits a uniform routine for all soft and hard tissues, especially cartilage; and it provides for numbers of tissues being taken from the same animal simultaneously and handled more satisfactorily.

Procedure. Tissues are taken from anesthetized animals, wiped with dry gauze and dropped quickly into ground glass-stoppered jars. After being cooled in a refrigerator to minimize water loss, the tissues that have visible connective tissue or fat are placed on a tile and adherent structures removed. The tissues are then dropped into glass-stoppered weighing bottles and again cooled before mincing with points of scissors. Tissues such as liver and brain that do not have visible fat or connective tissue are simply cooled and minced. Skin after being trimmed is cut into strips 8×10 cm. Samples are allowed to come to room temperature, and then weighed into vycor crucibles in aliquots depending upon amount of wet tissue available. These samples can range to 10 g of wet tissue. It is preferable to weigh 2 or more samples of the same tissue to check determinations for water and fat. The crucibles are next placed in 100°C thermostatically controlled oven for 48 hours, after which they are cooled in a desiccator over activated alumina oxide and then weighed. After this, the dried tissues are covered with dry ethyl ether and placed in another desiccator containing sufficient ethyl ether to cover bottom of desiccator to about one inch. After remaining in this ether desiccator for 24 hours, the ether layer over tissue containing extracted fat, is removed with Pasteur pipet and new dry ethyl ether added. This process is repeated at least 3 times. After removal of last ether extraction, the crucibles are placed in 100°C oven for approximately 5 hours, then cooled in a desiccator over activated alumina oxide and weighed. This gives a dry, fat-free solid which can be kept in a desiccator until ready to be ground and analyzed.

Grinding the dry tissue. The mills used for grinding dry tissues were either a Zassen-

* This work was supported by Grant A972 from N. I. of Arthritis and Metabolic Diseases, N. I. H., P.H.S.

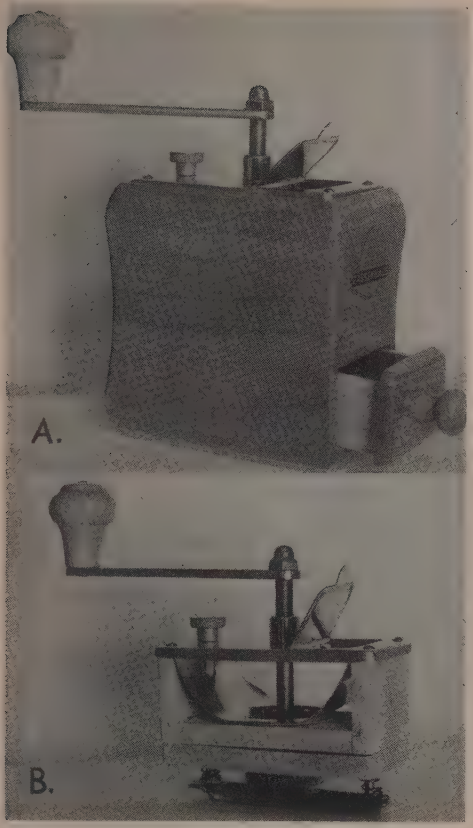

FIG. 1. A. Zassenhaus spice mill showing opened spring door through which tissue is introduced into mill and opened drawer that receives the ground sample. On top is also shown grinding handle and screw for setting size of desired particles. B. Cutting unit lifted out of mill.

haus spice or pepper mill,† depending on size or weight of tissue sample. These mills have hard steel cutters that can be adjusted for size of particles by a turn screw in top of spice mill or in bottom of pepper mill. The set screws on both ends of spice mill can be removed permanently so that the cutting unit can be lifted out readily. Illustrations of spice mill and cutting unit are shown in Fig. 1.

For grinding, dried tissue is scraped quantitatively with stainless steel spatula from the vycor crucibles onto a square of glazed paper and then transferred by brushing into the cutter compartment through a spring door on top of mill. The tissue is ground in a few minutes, requiring no effort. The powder is collected into a small drawer of the mill or in a devised cellophane bag. If the drawer is used, the powder from the drawer is transferred to a square of glazed paper, the cutters are lifted out and the drawer and the mill box are tapped sharply several times on the glazed paper until all particles of tissue have fallen from mill onto paper. The cutters are held over the glazed paper and any powder sticking to them is brushed with the sample for quantitative recovery. If the cellophane bag is used it is prepared as follows: a strip of heavy cellophane $18 \times 6''$ is wrapped around the cutter unit, using the extra for overlap; then bottom part of cellophane envelope is pleated and a hem is folded over to close the end of bag which is then fastened with a paper clip. For grinding, the cutting unit can either be returned to mill box for holding or it can be held firmly with a wood clamp attached to laboratory desk. When grinding is finished, cutter unit with attached cellophane bag is removed and placed on laboratory desk, the paper clip removed and the bag opened flat. Any adhering powder on cutters is brushed with the sample on the cellophane piece. The ground samples can be placed into small screwtop jars for later work, or they can be ground finer immediately.

To grind sample finer, samples from the mills are placed in a mullite mortar and either ground by hand or ground with a Fisher Mortar Grinder. The Fisher Electric Grinder is faster and superior to hand grind, requiring only about 15 minutes for soft tissues, and a little longer for cartilage samples. The powdered tissues are then returned to screw-top jars and, before weighing aliquots for analyses, they are dried in a 100°C oven overnight, cooled and kept in a desiccator over activated alumina oxide.

Summary. Improved procedures for preparation of tissues for chemical analyses have been presented. These procedures have definite advantages over all previous procedures; they are saving in both time and effort; they

† Kirkhams, Glens Falls, N. Y. Mill No. M495.

permit a uniform routine for all types of tissues; they produce homogenous materials that insure adequate sampling, and they enable numbers of tissues to be taken from the same experimental animal and handled satisfactorily.

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Received October 15, 1956. P.S.E.B.M., 1956, v93.

The Breeding System in *Stylonychia putrina.* (22829)

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Mating types have been reported in *Paramecium aurelia*(1-4), *P. bursaria*(5-7), *P. caudatum*(9-12), *P. multimicronucleatum*(8, 9), *P. trichium*(13), *P. calkinsi*(14), *Euplotes patella*(15), *Tetrahymena pyriformis*(16-18), *Stylonychia putrina*(19), and *Oxytricha bifaria*(20). In several of these species a number of sexually isolated varieties have been discovered. In some, the number of mating types found in each variety was 2, but in *P. bursaria, P. multimicronucleatum, P. trichium, E. patella, T. pyriformis, S. putrina,* and *O. bifaria* multiple mating types have been found. In one variety of *P. bursaria* there were eight; in *E. patella,* 6; in a variety of *T. pyriformis,* 11; in *S. putrina,* 5; and in *O. bifaria,* 9. The present paper reports another variety and additional mating types in the first variety of *S. putrina* reported in 1952 (19).

Materials and methods. Cultures of the 5 mating types in the first variety reported in 1952 died out in the laboratory. Two clones of progeny from crosses that had been made between clones of some of these 5 mating types were left. In 1952, 1954, and 1955, 155 additional clones of *Stylonychia putrina* were collected in Southern California in 6 different sites, 3 in the mountains and 3 in the valleys. The food was a liquid culture of a species of *Chlamydomonas* to which a loop of *Aerobacter cloacae* was added. The animals were isolated and grown in depression plates and later transferred to vials. The clones were fed every day, often twice a day.

Results. Mating behavior. The mating behavior found was that described previously (19), except that some degree of mating activity was observed in practically all of the 155 clones reported here, while none was observed in more than a third of the 41 clones reported in 1952.

Varieties and mating types. The two clones of progeny mentioned above reacted with some of the clones of a group that is called Variety I in this report. The mating types of Variety I now in existence have not been correlated with those previously reported. In 1954, six mating types were found in this variety. Various crosses were made among clones of these 6 mating types and among the progeny raised were 5 clones belonging to 3 new mating types. Among clones collected in 1955, 6 additional mating types were found, making a total of at least 15 mating types in Variety I. Eleven mating types were found among the Variety II clones, giving a total of 26 mating types for the two varieties.

The situation in *E. patella*(15) differed from that in *Paramecium* in that selfing was common in *Euplotes* and it was shown that some of the pairs found in conjugating mixtures consisted of 2 animals from the same clone. Also, animal-free fluid from a culture of one mating type induced selfing among animals of other mating types. Because of these differences, Sonneborn(3) concluded that the conception of a mating type in *Euplotes* was different from that in *Paramecium*. Selfing was uncommon in the 155 clones reported in this paper, but no method was found to determine the clonal origins of the animals associated in pairs. It is, therefore, not possible to state which of the two concepts of mating type applies to *S. putrina*.

Summary. Two sexually isolated varieties were found among 155 clones of *Stylonychia putrina* collected in Southern California plus 5 clones produced in the laboratory. In Variety I, at least 15 mating types have been found; in Variety II, 11 mating types.

The writer wishes to thank Dr. T. T. Chen for his help and guidance in this work.

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Received October 19, 1956. P.S.E.B.M., 1956, v93.

Influence of Steroids on Carbohydrate Content of Rabbit Kidney: A Chromatographic Study.* (22830)

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Intraglomerular kidney lesions produced in rabbits by the short-term administration of cortisone(1), prednisone and prednisolone are stained by the periodic acid-Schiff (PAS) method, a reaction of carbohydrates possessing free 1,2-glycol and amino alcohol groups (2). In the course of a systematic investigation into the nature of these renal lesions, chromatographic studies were designed to demonstrate the effects of cortisone acetate,† prednisone,‡ and desoxycorticosterone acetate on concentrations of certain carbohydrates extractable from rabbit kidneys. The sugars studied, rhamnose, mannose, fucose, glucose and galactose, are known to stain with PAS (2).

* This work was supported by Public Health Grants and by grant from Canadian Life Insurance Officers' Assn.

† Cortisone acetate was supplied through the courtesy of Mr. H. K. McLaughlin of Upjohn Co. of Canada.

‡ Prednisone was supplied through the courtesy of Mr. J. W. Brisick, Schering Corp. Ltd. of Canada.

Materials and methods. New Zealand white male rabbits ranging from 2 to 2.8 kg were maintained for a control period of 7 to 14 days on Sherwood rabbit pellets *ad libitum*. The left kidney was removed under nembutal and ether anesthesia. Postoperatively, the animals were provided with the same diet, but drinking water was replaced by 1% sodium chloride solution. This regimen was continued for 3 weeks during which the animals were divided into the following treatment groups: Group A: Control; Group B: 5 mg of desoxycorticosterone acetate intramuscularly/day; Group C: 5 mg of desoxycorticosterone acetate and 7.5 mg of cortisone acetate intramuscularly/day; Group D: 5 mg of prednisone suspension intraperitoneally/day. After 21 days, the animals were killed by overdose of nembutal and were autopsied. Portions of right kidney were fixed in Zenker-Formol, sectioned at 2.5 μ and stained with PAS stain. The remaining kidney tissue was extracted with 0.5 N sodium hydroxide for 4 days at 4°C; after neutralization, the extract was treated with 2 volumes of cold ethanol to precipitate acid mucopolysaccharides (fraction 1). A second precipitate (fraction 2) was obtained by increasing the ethanol concentration to 84%. Deproteinization of these extracts was not attempted. The 2 fractions were hydrolysed with a cation exchange resin (Permutit Q), and hydrolysates subjected to paper chromatography as described by Glegg and Eidinger (2). In this technic, hexosamines and hexuronic acid were either destroyed or adsorbed by the resin and did not appear on the chromatograms. After development of the chromatograms and location of sugar areas with aniline hydrogen oxalate, the quantities of the various sugars present were estimated by comparing their spot intensities under ultraviolet light (3660 Angstrom units) with those resulting from subjecting 125 μg of standard sugars to the chromatographic procedure. In this manner it was possible to make a crude estimation of the quantities of sugar in milligrams per hundred grams of fresh kidney.

Results. Several areas in all kidneys were observed to stain positively with the PAS technic: (a) capillary basement membrane of the glomeruli (b) the brush border of cells lining the proximal convoluted tubules and (c) the cytoplasm of cells lining the convoluted and collecting tubules. In the control group and in animals treated with desoxycorticosterone acetate alone, no further staining areas were identified. In the groups which received cortisone and prednisone, characteristic nodular glomerular lesions, some of which stained with PAS, were observed. A moderate increase in the quantity of PAS-staining material was noted in the capillary walls and in intratubular casts. These lesions were identical to those described by others(1,3). The pathological alterations were more numerous and severe in the group treated with prednisone.

Fig. 1 indicates the concentrations of rhamnose, mannose, fucose, glucose and galactose in fractions 1 and 2 in each of the groups. The most notable alterations were the disappearance of rhamnose from the kidneys of the groups treated with cortisone acetate or prednisone and a diminution in concentration in those treated with DCA. There was an increase in mannose and galactose content in all groups treated with steroids, but the levels of fucose and glucose were not significantly changed.

Discussion. Sommers and Haley(4) noted the accumulation of an abnormal substance in the glomerular stroma, capillaries, and walls of kidney arterioles in humans and ham-

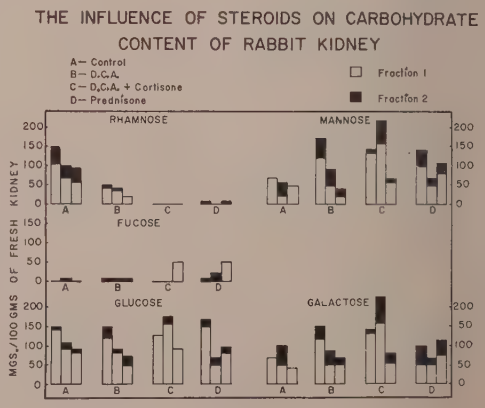

FIG. 1.

sters treated with cortisone. These changes are similar qualitatively to those which have been reported in human diabetic glomerulosclerosis(5). The abnormal substance was felt to be a mucopolysaccharide, since it is removed by incubation with hyaluronidase.

These findings coupled with those described above indicate an influence of adrenal steroids on the carbohydrate content of the kidney which may represent a link in the pathogenesis of glomerulosclerosis in human diabetes.

Summary. The changes induced in concentration of rhamnose, mannose, fucose, glucose and galactose in the kidney of rabbits treated with various steroids were studied using a semi-quantitative chromatographic method. The findings are discussed in relation to the pathogenesis of diabetic glomerulosclerosis.

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Received October 19, 1956. P.S.E.B.M., 1956, v93.

A New Colorimetric Reagent for Micro Determination of Ammonia.* (22831)

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This paper describes a new colorimetric procedure for the measurement of ammonia in quantities of 0.05 to 0.5 micromole. The method has proved to be more rapid and convenient than the usual microtitration, and the precision appears to be at least as good. The microdiffusion method of Conway(1) is employed to separate the ammonia from interfering substances. The subsequent measurement is based on the well-known reaction of ammonia with hypobromite. A carefully measured volume of hypobromite solution is added, enough so that an excess is present. The hypobromite remaining after the oxidation of the ammonia is then measured by its power to decolorize a dilute solution of phenosafranin. Previously described methods based on the reaction of ammonia with hypobromite depend upon iodometric titration of the excess hypobromite. In one such procedure, that of Levy and Palmer(2), as little as 0.36 micromole of ammonia is oxidized by hypobromite in a volume of 2 ml. For studies of ammonia in blood and tissues, however, it is desirable to work at still greater dilutions. As a first step, therefore, the iodometric method was used to test the reaction at greater dilutions. It was thus established that the oxidation of ammonia by hypobromite is stoichiometric at ammonia concentrations at least as low as .03 micromole per ml.

The choice of phenosafranin as a colorimetric reagent was based on tests of a number of dyes and stains. Although hypobromite has a decolorizing action on many such intensely colored substances, it was found that in most instances the decolorizing action is not directly proportional to the hypobromite concentration. With phenosafranin, however, a strictly linear relationship obtains.

Methods. Borate buffer, 0.5 *M,* pH about 9.8. A 16 g quantity of sodium hydroxide is dissolved in 800 ml water, 31 g boric acid is added and dissolved, and the solution is diluted to 1 l. *Phenosafranin,* approximately 0.006%. It is convenient to prepare a liter of 0.02% solution and to make dilutions as required. The concentration should be so ad-

* Supported in part by funds supplied by Wisconsin Alumni Research Foundation, and by Grant B-818 from N. I. of Neurological Diseases and Blindness of N. I. H., P. H. S.

justed that when 1 ml is added to 7.5 ml water and 1 ml of borate buffer, the optical density at 515 mμ is about 0.9. The phenosafranin solution is very stable. *Bromine solution.* While this can be prepared from liquid bromine and potassium bromide, a more convenient method is as follows: 16 g potassium bromide and 1.67 g potassium bromate are dissolved in 450 ml water. An 8.5 ml volume of 10 *N* sulfuric acid is added and the solution is diluted to 500 ml. It should be stored in a dark place. The bromine concentration slowly decreases. *Hypobromite reagent.* To 25 ml of the borate buffer is added about 0.5 ml of the bromine solution. The concentration should be so adjusted that when 1 ml of phenosafranin is added to a mixture of 2.5 ml water and 1 ml of hypobromite, with subsequent dilution to 9.5 ml, the optical density at 515 mμ is between 0.1 and 0.2. The hypobromite should be freshly prepared on the day of use. A slow change to the inactive bromate occurs, amounting to about 1% per hour.

Procedure. Microdiffusion is carried out as described by Conway(1), ammonia being absorbed in 1.5 ml of 0.01 *N* sulfuric acid in the central chamber. At end of diffusion period the contents of this chamber are transferred to colorimeter tube by a dropper. The chamber is washed with 1 ml water which is transferred to the colorimeter tube as completely as possible. To the solution in the tube (volume 2.5 ml) is added 1 ml of hypobromite solution. After at least 2 minutes, 1 ml of phenosafranin is added. After at least one minute, 5 ml of water is added. The solution is very thoroughly mixed after each addition. The optical density is read at 515 mμ. Results are calculated from a calibration curve prepared from standard ammonium sulfate solutions subjected to microdiffusion procedure. All readings are taken with instrument set so that distilled water gives an optical density of zero (100% transmittance). Blank determinations are run in triplicate with each group of samples, and for each ammonia determination the average blank value is subtracted from the reading. Care must be taken to insure that amount of ammonia in the aliquot of sample is less than the amount of hypobromite added, since the curve becomes a horizontal line when the hypobromite is completely reduced by ammonia (Fig. 1). If the reading approaches the upper limit, the determination should be repeated, if possible, on a smaller aliquot.

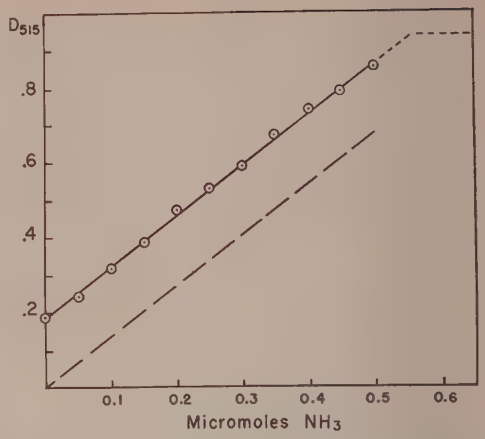

FIG. 1. Standardization curve. Horizontal portion at upper end of curve (dotted line) indicates color intensity of phenosafranin when no hypobromite is added, or when all of the hypobromite is reduced by ammonia. Dashed line represents curve after subtraction of the blank from each reading.

Results. Fig. 1 shows a standardization curve done on ammonium sulfate solutions directly, without microdiffusion. Readings were taken with a Coleman 6 B instrument. The dashed line represents the curve obtained after subtraction of the blank value from each reading. The points on the curve represent individual determinations done in one group, and the small deviations from the curve suggest the degree of precision which can be attained. The magnitude of the blank varies from one run to another, being dependent upon the concentration of hypobromite, but the slope of the curve is highly reproducible.

Application to brain tissue. The tissue (obtained after freezing the brain *in situ*) is ground and extracted with cold 10% trichloroacetic acid, using approximately 5 ml/g. After filtration, 1 ml aliquots of the extract are used for the determination. The microdiffusion must be started immediately, and is carried on for a 90-minute period. Correction for the slow formation of ammonia from glutamine during microdiffusion is made as

described by Richter and Dawson(3).

Application to blood. For measuring blood ammonia it is desirable to reduce the range of the method. The concentrations of hypobromite and of phenosafranin are reduced by about one-third. The volumes of reagents used are not changed, but the final dilution is made with only 1.5 ml water, giving a final volume of 6 ml. Blood is treated with ammonia-free potassium oxalate to prevent clotting, and the determinations are carried out on 1 ml aliquots. These may be pipetted into the Conway units and the diffusion started within 5 minutes after the blood is drawn, or more elaborate precautions may be taken to avoid ammonia formation after shedding(1). The diffusion period is limited to 20 or 30 minutes, in order to minimize ammonia formation due to action of the alkali on other blood constituents. The calculations are based on standards treated in the same way, since recovery is much below 100% in these short diffusion periods. Conway's(1) correction factors may be applied to allow for ammonia formation after shedding and for that due to action of the alkali, and for the slightly lower rate of diffusion from blood-carbonate mixtures as compared with water-carbonate standards.

Glutamine. The colorimetric method has been applied to the measurement of glutamine in brain tissue. One ml of the trichloroacetic acid extract is diluted with 4 ml of 10% trichloroacetic acid, stoppered loosely, and heated at 70° for 75 minutes. The glutamine is thereby converted to ammonia and pyrrolidonecarboxylic acid (method of Richter and Dawson(3)). The total ammonia is determined on 1 ml aliquots, and the value for preformed ammonia is subtracted.

Non-protein nitrogen. The method has been applied to the direct determination of non-protein nitrogen without microdiffusion. A suitable aliquot of a trichloroacetic acid filtrate of blood or tissue is digested by the Koch-McMeekin method with the precautions recommended by Miller and Miller(4). After the last addition of hydrogen peroxide, the heating is continued for 40 minutes to insure complete removal of hydrogen peroxide. (This is a necessary precaution, since any hydrogen peroxide remaining would react with the hypobromite). After cooling, the digest is diluted and enough sodium hydroxide is added to almost, but not quite, neutralize the sulfuric acid present. (The amount of sodium hydroxide necessary may be determined on a blank digest.) The solution is then made up to a definite volume and aliquots are taken for the determination. Blanks are run on the digestion procedure, and the calculations are based on standards not subjected to microdiffusion.

Summary. A new colorimetric procedure is substituted for microtitration in the measurement of ammonia by the Conway method. The ammonia is oxidized by addition of hypobromite, and the excess hypobromite determined by its power to decolorize phenosafranin. The range of the method as described is 0.05 to 0.5 micromole. The procedure has been applied to brain tissue and to blood, and to the measurement of glutamine. It has also been used for the determination of nonprotein nitrogen after Koch-McMeekin digestion, the microdiffusion being omitted.

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Received October 22, 1956. P.S.E.B.M., 1956, v93.

Ventricular Arrhythmias and Automaticity following Norepinephrine.* (22832)

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In human subjects norepinephrine was shown to increase peripheral vascular resistance without accelerating the heart rate(1). Norepinephrine has also been reported to be less effective in precipitating ventricular ectopic rhythms(2) and it is being used in treatment of secondary shock when epinephrine is contra-indicated to restore arterial blood pressure. Recently, norepinephrine was observed to increase the vulnerability of the ventricles to fibrillation and the greater safety reported for norepinephrine was considered to be due to its slower rate of infusion in clinical practice(3). Because norepinephrine is employed extensively in treatment of hypotensive states, this study was undertaken to compare its potency relative to that of epinephrine in eliciting experimental, ventricular rhythms. Using cats under pentobarbital, harman methosulfate was given to predispose the heart to ventricular rhythms by a method previously described(4). To extend the quantitative comparison of norepinephrine and epinephrine on cardiac excitability, their actions on isolated cardiac papillary muscle preparation of Cattell and Gold(5) were also investigated.

Methods. Experimental ventricular arrythmias. The electrocardiographic changes produced by epinephrine and norepinephrine were studied in normotensive cats under sodium pentobarbital anesthesia. Harman methosulfate (2.5 mg/kg) was injected 5 minutes before epinephrine or norepinephrine and the ensuing electrocardiographic changes were recorded. *Automaticity studies.* Following the technic of Cattell and Gold(5) isolated, cat, cardiac papillary muscles were mounted in lucite holder and immersed in 75 ml of modified Locke's solution(6). The chordae tendinae were connected by a fine silk thread to a thin steel plate on each side of which a Baldwin SR-4 strain gauge was mounted and contractions were recorded with the strain gauge amplifier of a Sanborn Twin-Viso Cardiette. The muscle was stimulated electrically with a square wave shock from a thyratron stimulator at 60 per minute and the amplitude of contractions and threshold of excitability were noted. The incidence of automaticity (rhythmic contractions without electrical stimulation) was observed following addition of norepinephrine and epinephrine with equilibration period of at least 15 minutes between drugs.

Results. Nodal rhythm (NR), ventricular extrasystoles, tachycardia and flutter (VR) and ventricular fibrillation (VF) occurred following injection of both epinephrine and norepinephrine in cats previously treated with harman methosulfate (Table I). Immediately before the onset of fibrillatory rhythm, a migration of the T-wave into the QRS could be seen (Fig. 1). A similar interruption of T-waves by R-waves was observed by Fastier to occur before "epinephrine syncope" following benzene or furfurin(7). In contrast to larger mammals, cats were able to survive relatively long periods of ventricular fibrillation. Based on both the duration of arrhythmias and the incidence of ventricular fibrillation, norepinephrine was found to be significantly more active than epinephrine in eliciting ventricular ectopic rhythms ($p < 0.05$). *Automaticity of papillary muscles.* A pos-

TABLE I. Incidence and Duration of Arrhythmias in Cats Treated with 2.5 mg/kg Harman Methosulfate.

Drug	Dose, μg/kg	Number				Duration of arrhythmias, sec.*
		Total	NR	VR	VF	
Epinephrine	2.5	10	0	6	3	8.4± 3
	5.0	10	2	9	6	54 ±16
	10.0	11	3	11	5	78 ±20
	20.0	10	4	10	7	66 ± 9
Norepinephrine	1.2	10	1	10	4	82 ±43
	2.5	10	0	9	4	116 ±40
	5.0	10	3	10	6	198 ±30
	10.0	10	4	10	9	162 ±16

* Mean ± S.E.

* This study was supported in part by grant from National Research Council of Canada.

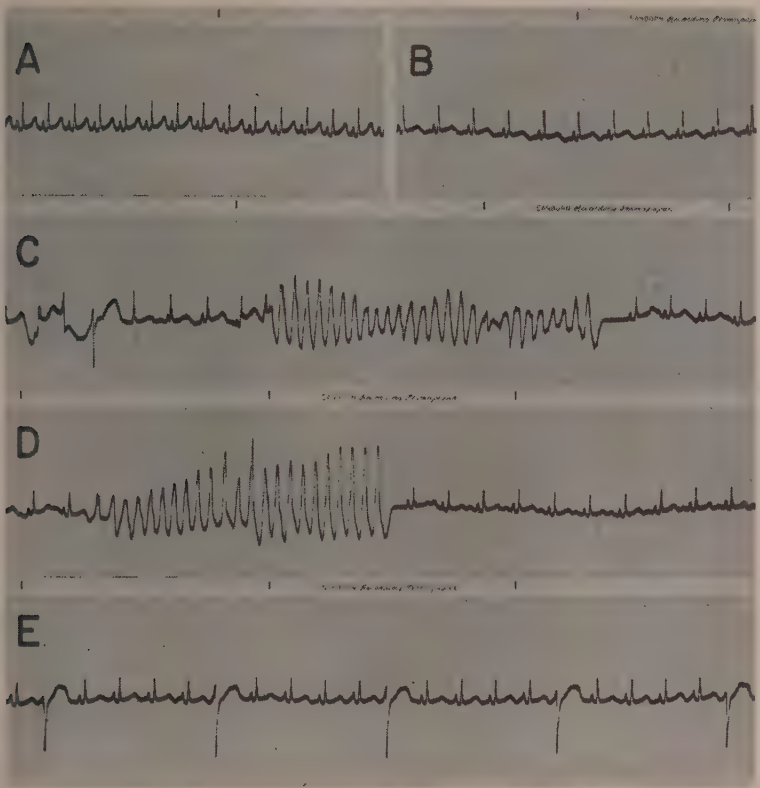

FIG. 1. Electrocardiograms of cat (2.4 kg) under sodium pentobarbital. A, 11:37 a.m., control reading. B, 11:44 a.m., 4 min. after harman methosulfate 2.5 mg/kg intrav. C, 11:45 a.m., 12 sec. after norepinephrine bitartrate 5 μg/kg intrav. D, continuation of C. E, 11:47 a.m., 2 min. after norepinephrine bitartrate.

sible relationship between ability of a substance to initiate automaticity and the property of precipitating ventricular ectopic beats was previously suggested (4). A comparison of the incidence of automaticity in the papillary muscles after addition of epinephrine and norepinephrine was therefore indicated to see whether norepinephrine was also more active in this preparation. As shown in Fig. 2, norepinephrine was found to be effective in increasing contractile force and inducing rapid, automatic contractions of papillary muscle. Although changes of contractile force of the papillary muscle produced by norepinephrine and epinephrine did not differ, the incidence of automaticity after norepinephrine was significantly greater than after epinephrine ($p < 0.05$).

TABLE II. Effects of Epinephrine and Norepinephrine on Cardiac Papillary Muscles.

Drug	Conc.	Incidence of automaticity	Mean % increase in amplitude of contractions
Epinephrine	$.4 \times 10^{-6}$	2/12	60
	1.6 "	6/12	123
Norepinephrine	.2 "	5/12	66
	.8 "	8/ 9	95

To compare the cardioaccelerator properties of epinephrine and norepinephrine with their effects on cardiac excitability, the increases in heart rate in isolated, perfused cats' hearts were determined. The tachycardia following epinephrine was greater than for equivalent doses of norepinephrine although the difference in activity was not significant. Thus, the relative effects of these substances

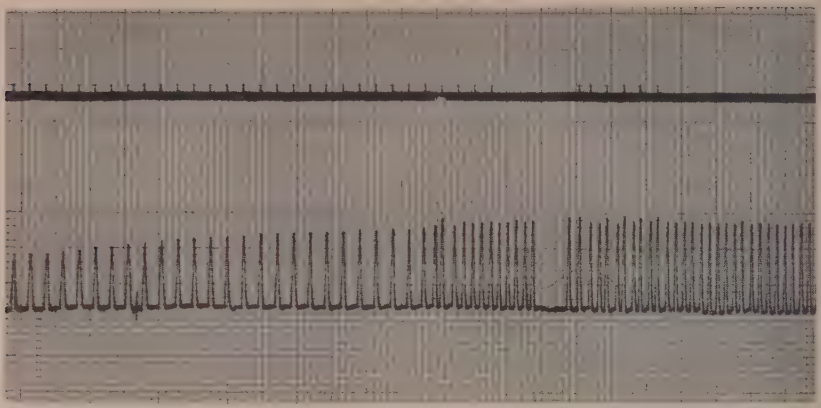

FIG. 2. Increase in contractile force and automaticity induced by norepinephrine bitartrate (1.6×10^{-6}) in the cat's heart papillary muscle. Upper record, stimulus marker (frequency, 1/sec.; duration, 10 millisec.; 15 volts). Lower record, contractions recorded by a strain gauge and amplifier of a Sanborn Twin-Viso Cardiette. Five horizontal lines represent 1 g.

on the sinus node and on ventricular excitability appeared to be separable.

Discussion. Electrocardiographic changes consisting of heart block and supraventricular rhythms were occasionally observed in normal subjects after intravenous norepinephrine(9). In cases of shock, on the other hand, most authors have found no evidence that the drug increased myocardial irritability during the hypotensive phase even after coronary occlusion. Nevertheless, the high potency of norepinephrine in precipitating ventricular rhythms in normotensive cats treated with harman methosulfate as well as in increasing cardiac irritability(8) points to a potential hazard of this pressor amine in clinical practice. Both epinephrine and norepinephrine were found to be less likely to elicit ventricular rhythms including fibrillation in dogs under cyclopropane which were made hypotensive by bleeding(2). The failure of norepinephrine to provoke arrhythmias in the therapy of shock in which the heart is vulnerable to fibrillation may therefore be related to the low blood pressure levels in these cases and not to the fact that norepinephrine lacks the effects of epinephrine on ventricular excitability.

Summary. (1) Nodal rhythm, ventricular extrasystoles, flutter and fibrillation were precipitated by norepinephrine in cats, under pentobarbital anesthesia, treated with harman methosulfate. Migration of the T-wave into the R-wave at onset of ventricular fibrillation was similar to that described before epinephrine induced arrhythmias. (2) The effects of norepinephrine on cardiac excitability were confirmed using isolated papillary muscles in which norepinephrine had a greater potency than epinephrine in eliciting automatic contractile activity.

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Received October 25, 1956. P.S.E.B.M., 1956, v93.

Serum Transaminase Activity in X-Irradiated Rabbits. (22833)

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Increased levels of serum glutamic oxaloacetic transaminase (G-O-T) activity after crushing and tourniquet injury to the rabbit leg were observed in our laboratories and will be reported elsewhere. Preliminary studies indicated that x-irradiation of left chest wall of an otherwise completely shielded rabbit in dosages of 2,500 r and higher, resulted in serum G-O-T elevations as early as 3 hours' post-exposure. It became of immediate interest, therefore, to determine if this very early response could be identified in rabbits after low dosage, whole body x-irradiation.

Methods. Rabbits of New Zealand strain, as in previous studies of traumatic injury(1), were employed. The animal, tied to a board, was placed 135 cm from center line of tube head. Dose levels of 500, 750, and 1,000 r were studied; half the dose was given ventrally and half dorsally in each instance.

Results. At the 500 r level, total irradiation time was 16.1 minutes. In another experiment, an attempt was made to determine if any relationship existed between initially elevated serum G-O-T activity and survival time of the animal.

The combined effects of bleeding and restraint on serum G-O-T activity were recorded in sham controls tied to the board in radiation position, without exposure, and bled serially along with x-rayed rabbits. Bleeding was accomplished by incising the ear vein, and transaminase determinations(2) were made on unhemolyzed sera after separation by low-speed centrifugation. The results are listed in Table I.

It is apparent that bleeding and restraint induce a transitory serum G-O-T activity increase that returns to normal values at 24 hours. However, the data demonstrate that a significant increase in serum G-O-T activity can be identified in the rabbit within 6 hours after 500 r of x-irradiation. At the 750 r and 1,000 r dose levels this alteration was recorded at 3 hours post exposure.

Perhaps the most striking aspect of transaminase change is its discriminating power on an individual basis. Of the 25 animals in the 750 r and 1,000 r experimental groups, 20 survived through 6 hours. In 15 of these 20, the 6-hour G-O-T activity ranged from 23 to 189 units above pre-irradiation levels. In 5 rabbits that died between 3 and 6 hours post-irradiation, the 3-hour determinations ranged from 22 to 196 G-O-T activity units above pre-irradiation values. In 15 sham control rabbits, on the other hand, the maximum change from "pre-irradiation" values at 6-hour sampling was 26 units (one animal) —the next highest change was 17 units (also a single rabbit). At the 500 r level, the significantly increased mean value for the group at 6 hours post-irradiation is not associated with much individual discrimination. Here, only 3 out of 12 rabbits recorded 6-hour G-O-T activity increases in excess of 17 units, although another 5 animals fell in the 14-16 units increase range.

The data in Table II indicate that there exists little relationship between the serum G-O-T change after 625 r of x-irradiation and survival time. The increase of 59 units, within the first 24 hours, recorded for rabbit No. 6 was associated with death on 8th day post-exposure. However, rabbit No. 9 died on 13th day with an observed change of −5 G-O-T units, while animal No. 5 survived the full 30-day term after an observed 24 unit increase within the first 24 hours. It would seem that although the initially elevated serum G-O-T activities may have resulted from increased permeability of irradiated tissue cell membranes, and thus more or less quantitatively reflect the extent of tissue damage, survival of the animal depends, in the main, on its ability to overcome and repair the insult.

Summary. Rabbits were subjected to x-

TABLE I. Serum Transaminase—Pre-irradiation and at Intervals Post-exposure. Means and standard errors.

	Pre-irradiation		+ 3 hr		+ 6 hr		+ 24 hr	
	N	Units/ml	N	Units/ml	N	Units/ml	N	Units/ml
Sham controls	15	22 ± 1.3	15	35 ± 2.2	15	30 ± 2.2	15	24 ± 1.7
500 r	12	24 ± 0.9	12	33 ± 1.3	12	38 ± 2.7*	11	31 ± 2.7*
750 r	12	22 ± 2.7	12	65 ± 14.1*	9	50 ± 5.3*	6	45 ± 7.9*
1,000 r	13	26 ± 1.7	12	52 ± 5.6*	11	66 ± 13.5*	8	42 ± 3.7*

* Difference statistically significant. Difference between means of sham controls and experimentals exceeds the stand. error for the difference by 2 + times.

TABLE II. Serum Transaminase and Time of Death (625 r Total Body Exposure).

Rabbit No.	Pre-irrad., units/ml	Max value 6-24 hr post-irrad., units/ml	Δ, units/ml	No. days 'til death
1	36	40	+ 4	12
2	26	50	+24	S* 30 d
3	30	50	+20	S 30 d
4	25	73	+46	21
5	26	51	+25	S 30 d
6	28	87	+59	8
7	29	43	+14	7
8	26	39	+13	S 30 d
9	38	33	− 5	13
10	15	50	35	8

* S = Survived.

irradiation in dosages of 500, 750, and 1,000 r. Serum glutamic oxaloacetic transaminase estimations were accomplished at 3, 6, and 24 hours post-irradiation. A significant increase in G-O-T activity could be identified at 6 hours in the rabbit group subjected to 500 r, and as early as 3 hours post-irradiation in the 750 r and 1,000 r animal groups. For the latter animals, the noted increase at 6 hours served to segregate 75% of the group on an individual basis. In rabbits subjected to 625 r of x-irradiation no relation was found between the serum G-O-T elevation and survival time.

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Received October 26, 1956. P.S.E.B.M., 1956, v93.

Pathogenesis of Experimental Arteriosclerosis in the Rat.* (22834)

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We have demonstrated that the standardized production of severe arteriosclerosis (Mönckeberg type) and myocardial necrosis by means of renal injury in the albino rat can be completely prevented by prior thyroparathyroidectomy(1). Studies directed towards elucidation of the mechanism underlying this striking protective effect of thyroparathyroidectomy, led to the conclusion that the cardiovascular injury of intact rats was caused primarily by autointoxication with parathyroid hormone. On further analysis it was inferred that excess function of the parathyroid glands following standard renal damage was mediated through the adrenal cortex. This communication describes experiments which formed the basis for these conclusions, and outlines our present concept of the mechanism of experimental arterial mediosclerosis.

Procedures. The method used for production of lesions has been described previously (2). The procedure consists in a single par-

* This investigation has been aided by grant H-890 (C5) of N. Heart Inst., N.I.H., U.S. Public Health Service.

enteral injection of the sodium salt of a poorly soluble sulfonamide in excessive dosage. This induces invariably massive and long-lasting intrarenal deposition of sparingly soluble crystals and results in obstructive nephropathy and permanent kidney damage, followed by severe disseminated cardiovascular and smooth muscle lesions in 4 to 7 days. The pathologic-anatomic changes are readily recognizable with the naked eye. Rats with typical injury manifest enlargement of parathyroids and adrenals as well as atrophy of thymus gland. Animals permited to survive for longer periods (2 to 5 weeks) show apparently no substantial progression of the injury but correspondingly increasing degrees of reparative changes in damaged organs such as scar formation and intense calcification of necrotic areas. Since emergence of these severe cardiovascular lesions could be obviated by prior thyro-parathyroidectomy, and since, in preliminary observations, a similar protective effect was achieved by adrenalectomy(3), further analysis of the mechanism responsible for disseminated muscular necrosis concerned the relative importance of the separate thyroid and parathyroid hormones and their possible relationship to the adrenal cortex. Three hundred adult male and female albino rats were used. In addition to thyro-parathyroidectomy, hypophysectomy and nephrectomy, the following procedures were carried out: Surgical removal of parathyroids and adrenals separately as well as in combination, elective and graded hormone substitution in such animals and in thyro-parathyroidectomized rats, and finally chemical "thyroidectomy," using 0.01% propylthiouracil in drinking water for a minimum of 4 weeks preceding the experiment. For hormonal substitution or treatment, cortisone and desoxycorticosterone acetate were injected subcutaneously in daily dosages of 3 mg each, starting 3 days prior to initiation of renal injury and continuing for a minimum of 3 days thereafter. Similarly, parathyroid extract was injected subcutaneously for substitution or poisoning in daily dosages ranging from 10 to 200 International Units, starting 5 days prior to kidney block and continuing for at least 5 days thereafter. Serial microdeterminations of calcium(4) and inorganic phosphate(5) were done in plasma of rats from most experimental groups. Complete postmortem examinations were performed on every animal. All important organs were fixed in formalin and sectioned for microscopic study using routine and special stains.

Results. Distribution of 9 sub-groups comprising 215 rats with various hormonal deficiencies and type of hormone employed are shown in Table I (Groups 2-11). For conciseness, experimental results are presented only for medionecrosis and calcification of the arterial tree as the severest and most consistent lesion (93% in intact rats, Group 1). However, data on vascular damage are fully in line with those for muscular necrosis in heart and gut.

Table I shows that treatment of thyro-parathyroidectomized rats with parathyroid extract in amounts of 50 units daily induced typical vascular damage (Group 3). Since even the smallest dosage (10 units) raised plasma calcium levels to normal and allowed for development of nephrocalcinosis, parathyroid substitution was believed to be more than adequate so that these rats could be considered as thyroidectomized only. It is clear, therefore, that vascular necrosis did occur in complete absence of the thyroid gland. Similarly, chemical "thyroidectomy" did not prevent vascular injury, although it did provide a substantial reduction of its incidence (Group 4). The lowered incidence was interpreted as due to diminution of parathyroid hormone formation in the absence of thyroid hormone.

The singular significance of parathyroid hormone in pathogenesis of arterial necrosis is illustrated by the full protection afforded the 30 rats of Group 5 by parathyroidectomy alone. Moreover, one can readily enlarge this number by adding the 29 rats of Group 2, and, with some reservations, the 14 animals of Group 9. Thus, none of a total of 73 parathyroprive rats showed vascular lesions or other muscular necrosis under careful microscopic examination. Exhibition of parathy-

TABLE I. Hormonal Influences upon Development of Renogenic Arterial Medionecrosis in Albino Rats. (215, 4- to 6-mo-old animals of both sexes, with standard renal injury.)

Group No.	Endocrine status prior to hormone admin.	Hormone treatment or substitution daily	No. in group ♂	No. in group ♀	No. in group Total	No. with arterial necrosis	Incidence of arterial necrosis, %
1	Intact	—	31	15	46	43	93
2	TPX	—	24	5	29	0	0
3	TPX	PE (10-20 units)		5	5	0	
		PE (50 ")		5	5	2	
4	"TX"	—	4	8	12	3	25
5	PX	—	22	8	30	0	0
6	PX	PE (100 units)	18		18	13	72
7	AX	Cortisone	18	6	24	0	0
8	AX	Cortisone & DCA	10	10	20	12	60
9	AX & PX	*Idem*	7	7	14	0	0
10	AX	Cortisone & PE (200 units)	12		12	8	67

X = Removal of gland, T = Thyroid, P = Parathyroid, A = Adrenal.
PE = Parathyroid extract, DCA = Desoxycorticosterone acetate.

roprive rats to excess amounts of parathyroid extract (100 I.U. daily) restored their ability to develop the characteristic lesions, as depicted in Group 6. Of 18 rats in this group, 13 or 72% presented typical vascular damage.

It was postulated, therefore, that autointoxication with hormone from excessively secreting parathyroid glands was responsible for emergence of muscular necrosis in intact rats with renal injury, (Group 1). Further evidence was seen in behavior of plasma calcium and phosphate levels of these animals in the initial stages of renal disease, in which calcium levels were normal or even elevated (9-15 mg%) in the face of marked retention of inorganic phosphate (8-12 mg%).

In further pursuing the role of parathyroid gland, rats deprived of their adrenals and maintained on cortisone did not incur parathyroid hypertrophy, and, like parathyroidectomized rats, failed to develop the characteristic picture of disseminated muscular necrosis (Group 7). However, when adrenalectomized animals were given DCA in addition to cortisone (Group 8), the parathyroid glands became greatly enlarged and typical cardiovascular and smooth muscle necrosis appeared. (Almost identical observations were made with hypophysectomized rats, not shown in Table I.) On the other hand, when adrenalectomized animals were, in addition, deprived of their parathyroid glands, DCA administration appeared to have lost its deleterious effect (Group 9), whereas typical muscular injury could be readily produced in adrenalectomized rats given parathyroid extract (200 I.U. daily) *instead* of DCA (Group 10).

Under our experimental conditions, therefore, desoxycorticosterone administration in itself to rats with standard renal injury does not induce arterial necrosis; rather an ample supply of adrenal steroid of this type seems essential for development of hyperparathyroidism which, in turn, causes vascular injury. Hence a hitherto unknown direct or indirect stimulatory action of mineralocorticoids upon the parathyroid gland must be postulated. Preliminary evidence indicates that, to a certain extent, androgens can enhance this desoxycorticosterone effect, whereas estrogens cannot. In fact, female sex hormone which has been shown to be capable of inhibiting the output of adrenal steroids(6), appears to counteract the development of muscular necrosis.

In line with this observation, the incidence and severity of arteriosclerosis in the young adult rat were found to be substantially higher in male than in female animals. This difference was most pronounced in 2-month-old animals, and gradually decreased with advancing age of the rat. In 2-month-old rats (*not used in this study*), incidence of lesions is around 80% in the male rat and around

TABLE II. Influence of the Parathyroid Gland upon Development of Cardiovascular Necrosis in Nephrectomized Albino Rat. (Male animals, 4-6 mo old.)

Group	Description of procedures	Rat No.	Days of survival post-nephrectomy	Macroscopic pathology: Necrosis of Myocardium	Macroscopic pathology: Necrosis of Aortic media
A	Control, nephrectomy only	1	3	—	—
		2	2	—	—
		3	4	—	1+
		4	3	4+	2+
		5	3	1+	1+
		6	3	1+	—
		7	3	2+	1+
		8	3	1+	1+
B	Parathyroid extract, 100 I.U. s.c., on 1st and 2nd day post-nephrectomy	9	3	3+	3+
		10	3	4+	1+
		11	3	4+	3+
		12	3	4+	3+
		13	3½	4+	2+
C	Parathyroidectomy, 1 wk prior to nephrectomy	14	3	—	—
		15	3	—	—
		16	4	—	—
		17	4	—	—
		18	5	—	—

Definition of lesions: —, none; 1+, mild, localized; 2+, moderate and more extensive; 3+, severe and widespread; 4+, maximal degree

50% in the female rat. In the 4-6-month-old group, male rats show an incidence of 95% of mostly severe aortic lesions, compared with an incidence of 70% of moderate to severe lesions in female animals.

In our most recent studies, involving 10 adrenalectomized, 15 parathyroidectomized and 50 nephrectomized rats, the following additional facts were established: (1) It is possible to induce marked hypertension and the characteristic picture of disseminated necrosis in heart, arterial tree and gut in parathyroidectomized rats *not* subjected to standard renal injury, by daily administration of 100 to 200 units of parathyroid extract. These animals may also develop marked nephrocalcinosis. (2) Adrenalectomized rats maintained on saline and not exposed to standard renal injury, manifest invariably pronounced systolic hypertension within 24 hours following injection of 100 units of parathyroid extract with pressures ranging from 160-210 mm Hg. Daily reinjection results in maintenance of hypertension except for a transient marked dip in blood pressure following each administration and lasting about 6 hours. (3) Cardiovascular necrosis and hypertension can likewise be produced in the rat by nephrectomy (Table II, Group A), which speaks against the importance of a renal factor in their development. The organic changes are closely similar to those induced by obstructive nephropathy and not unlike the pathologic-anatomical picture observed by Grollman and associates(7) and others in renoprival animals. (4) Severity of cardiovascular necrosis and hypertension produced by nephrectomy can be substantially enhanced by administration of parathyroid extract (100 units) on first and second day following removal of kidneys (Table II, Group B). (5) When parathyroprive rats are nephrectomized, they do not develop any lesions in the myocardium or the arterial tree recognizable under magnifying glass, although they seem to survive somewhat longer than rats with intact parathyroid glands (Table II, Group C).†

Comments. The concept of the mechanism leading to cardiovascular necrosis and calci-

† (The nephrectomy studies, exemplified in Table II, were carried out in cooperation with Dr. Isabelle Wajda. The results have since been fully duplicated and will be published elsewhere in detail.)

fication which emerges from our studies is briefly as follows: The "stress" of obstructive nephropathy causes adrenal cortical hypertrophy. Increased output of desoxycorticosterone-like hormones probably triggered primarily by severe derangement of mineral metabolism, results in pronounced stimulation of the parathyroid glands. Autointoxication with parathyroid hormone, in turn, is responsible for the picture of disseminated cardiovascular necrosis and calcification and substantially enhances renal injury (nephrocalcinosis). Concomitant "renal" hypertension probably contributes to the development of vascular lesions. In the light of this concept, it is understandable that mechanisms or procedures which tend to interfere with the availability of the desoxycorticosterone-like steroids, may result in a diminution of the extent and incidence of cardiovascular necrosis, whereas an increase in supply of such steroids may have the opposite effect.

These animal-experimental findings lead us to believe that in human disease as well the parathyroid gland may play a more important role in pathogenesis especially of renogenic cardiovascular necrosis and hypertension than is commonly assumed. For example, it is well known that rapidly progressive malignant hypertension, usually distinguished by extensive medionecrosis of small arteries, is often dramatically arrested by adrenalectomy(8,9). Credit customarily goes to removal of excess mineralo-corticoids and the consequent decrease of hypertension. The possible presence of a hyperfunctioning parathyroid gland is usually considered disproven by the finding of normal blood calcium levels, although it should be realized that, in subacute and chronic phases of clinical as well as experimental hyperparathyroidism, blood calcium levels may return to within the normal range. Moreover, one cannot exclude a possible inhibitory effect of adrenalectomy upon output of a specific vasoactive parathyroid factor, claimed to be separable from the calcium mobilizing activity of parathyroid extract(10) and responsible for both spasm and necrosis of muscles. Such a vasoactive principle may, in fact, represent the long sought extrarenal pressor principle held responsible for both renal and renoprival hypertension and known to be dependent upon adequate adrenal cortical activity(11). Finally, presence of anatomically normal parathyroid glands should not be relied upon as evidence against hyperfunction since, under physiological conditions, these glands were shown to operate only at a small fraction (5-25%) of their full capacity(12). This wide margin of safety allows for excess secretion without alteration of anatomical structure(13). It is conceivable, therefore, that subtotal parathyroidectomy may be more effective than the far more radical adrenalectomy in arresting rapidly progressive sequellae of severe renogenic hypertension.

Our data do not support the time-honored contention that in hyperparathyroidism, calcium deposition occurs in *normal* tissue, so-called "true metastatic calcification" in contradistinction to dystrophic calcification(14), since in our experimental animals calcium imbibition was invariably preceded by disseminated necrosis. Autopsies performed at predetermined stages of the experimental disease proved beyond doubt that areas of necrosis were those which later became calcified. It is of special interest in this connection that in a recently reported post mortem examination of a patient who died from renogenic hyperparathyroidism, the myocardium was found to contain foci of fresh necrosis, a fact which in the authors' view was the reason for extreme severity of "metastatic calcification" (15). Significantly they state that the cause for necrosis is obscure. In the more chronic phase of hyperparathyroidism when healing predominates, especially following various therapeutic efforts, necrosis may be absent, so that foci of calcification appear embedded in otherwise normal tissue. This is the very situation most commonly encountered at autopsy in human beings. It is probably responsible for the concept of "metastatic calcification."

Finally, it would appear in the light of our findings that a number of experimental lesions ascribed in particular to direct effects of desoxycorticosterone and saline(16) or a com-

bination of these with renal insufficiency (17) may deserve reevaluation.

Summary. 1. Pathogenesis of severe arteriosclerosis and disseminated myocardial necrosis, induced in the albino rat by standard renal injury, was investigated. These cardiovascular lesions are due to autointoxication with parathyroid hormone, and this hormone apparently contains an important hypertensive factor. 2. Evidence was presented that excess production of parathyroid hormone, as a consequence of renal dysfunctions, is mediated through the adrenal cortex and that mineralocorticoids are essential for direct or indirect activation of the parathyroid gland. To some degree, androgens can enhance the mineralocorticoid effect, whereas estrogens seem to inhibit emergence of muscular necrosis. The incidence of cardiovascular injury in intact and adrenalectomized rats was found to be substantially higher in male than in female animals. 3. It was shown further that, in the absence of parathyroid glands, mineralocorticoids in excessive dosage fail to produce cardiovascular necrosis, whereas excess parathyroid hormone assures emergence of typical lesions in complete absence of mineralocorticoids. In fact, by providing abundant supplies of parathyroid extract, cardiovascular necrosis was induced without renal injury and even in absence of kidneys. 4. Implications of these findings upon the concept of "metastatic calcification" and upon pathogenesis and therapy of renogenic cardiovascular necrosis and hypertension in human beings are discussed. It is suggested that subtotal parathyroidectomy be tried in preference to adrenalectomy in attempts to arrest rapidly progressive sequellae of malignant hypertension.

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Received July 19, 1956. P.S.E.B.M., 1956, v93.

Gastric Iodide and Chloride Clearances in Dogs.* (22835)

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Inorganic iodide is concentrated in gastric juice of experimental animals(1-4) and human beings(5-8) as effectively as in the thyroid gland(9). Investigation into the mechanisms was complicated by injection of sodium iodide(1,2) which partially interfered with the iodide-concentrating process(1). Several authors(1,2) have suggested that iodide might replace chloride in gastric juice but investigations regarding secretory drugs(3,4) have not included quantitative chloride and iodide data that would test the possible interchange of iodide and chloride.

Plasma clearances of iodide by the stomach have been determined in patients(8) and in rats(10). The iodide and chloride clearances were considered satisfactory quantitative indices of gastric secretory mechanisms. The present investigations were undertaken to compare these functions under the influence of substances known to alter the iodide trap of the thyroid gland(11). It was considered that clearance studies might give a basis for comparison of iodide and chloride secretory mechanisms of the stomach and some comparison of the gastric and thyroid iodine trapping mechanisms.

Methods. Acute experiments with Nembutal anesthesia: Mongrel dogs were fasted 6-18 hours before the experiment. They were lightly anesthetized by intraperitoneal injection of 1 cc 5.5% Nembutal per 5 lb. The femoral veins were exposed on both sides, one for injections and the other for blood sampling. The stomach was exposed by a midline incision, the esophagus tied with strong cord, and the pylorus cannulated by tying into place a thick rubber tube introduced into the stomach through the duodenum. Then 15 to 90 μc of I^{131} were injected intravenously. After a 10-30 minute period for mixing, the stomach was washed with approximately one liter of warm water in 3-4 portions and then returned to its normal position in the abdomen. After 7.5 minutes a blood sample was taken and after 7.5 additional minutes the stomach's secretions were collected by washing with a liter of water in 3 or 4 equal portions. This procedure was repeated in order to have 2 control collections after which the animal was injected with either Histamine (3 mg), NaSCN (75 mg/kg), NaI (60 mg/kg), or acetazoleamide (Diamox, .5-1 g). After 10-30 minutes, the stomach was washed and 2 collections of gastric secretions made and a blood sample taken at the mid-time of each. The gastric secretions and blood were analyzed for I^{131} by scintillation counting. Chloride was determined mercurimetrically(12). Gastric iodide and chloride clearances were determined as follows: $\frac{\text{Gastric } I^{131} \text{ or chloride/min.}}{\text{Plasma } I^{131} \text{ or chloride/cc}}$. *Chronic experiments on unanesthetized dogs with Heidenhain or innervated total gastric pouch:* The dogs were fasted 18 hours preceding the experiment, and allowed water *ad libitum*. A venous blood sample was drawn preceding the experiment and analyzed for radioactivity to determine residual activity left from previous experiments. If activity was found, PBI^{131} was determined; this never exceeded 3% of the total plasma I^{131}. Ten to 40 μc of I^{131} were injected into the left leg vein. After a 30 minute period for mixing and equilibration, the pouch was washed with 200 to 300 cc of warm water, using 60 cc washing. Seven to 8 minutes later a blood sample was taken from the right leg and at 15 minutes a gastric collection was made by washing the stomach with 200 cc of water in 60 cc portions. After the 2 control collections, the dogs were injected with Histamine (3 mg), NaSCN (75 mg/kg), $NaClO_4$ (15 mg/kg), NaI (60-100 mg/kg) or reserpine (.25 mg/

* The study was supported by research grants from the USPHS and USAEC. The gastric pouches were prepared through cooperation of Dr. E. H. Storer and his staff of Department of Surgery.

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TABLE I. Gastric Clearance of Iodide and Chloride.

Procedure	No. of exp.	Gastric clearance of plasma, cc/min. Iodide	Chloride
Acute exp., Nembutal anesthesia			
Control	6 (3 dogs)	4.7 (2.4-7.5)*	.40 (.17-.67)
Sodium iodide, 60 mg/kg		.53 (.28-1.0)	.35 (.13-.61)
Control	4 (2 ")	3.4 (3.0-4.3)	.46 (.33-.74)
Thiocyanate, 75 "		.39 (.32-.41)	.26 (.20-.30)
Control	8 (4 ")	7.3 (4.3-10.2)	.27 (.12-.46)
Histamine, .1-.14 "		9.9 (7.3-12)	.66 (.24-1.3)
Control	6 (3 ")	4.2 (3.1-5.3)	.26 (.16-.32)
Diamox, .5-1.0 g		4.9 (2.9-6.4)	.24 (.15-.30)
Unanesthetized female Heidenhain pouch dog			
Control	2	5.4 (4.8-6.0)	.27 (.21-.34)
Sodium iodide, 60 mg/kg		.70 (.60-.80)	.12 (.10-.14)
Control	2	4.4 (4.1-4.7)	.11 (.10-.12)
Thiocyanate, 75 "		.25 (.25-.45)	.12 (.10-.15)
Control	2	1.5 (1.3-1.6)	.12 (.11-.14)
$NaClO_4$, 15 "		.11 (—)	.12 (.11-.12)
Control	2	4.5 (4.4-4.5)	.10 (—)
Histamine, 3 mg		12 (11-14)	1.2 (.94-1.5)
Control	2	5.2 (4.8-5.7)	.25 (.24-.26)
Serpasil, 2.5 "		12 (9.5-14)	.87 (.57-1.2)
Male Heidenhain pouch dog			
Control	2	5.4 (4.6-6.3)	.10 (—)
Na I, 100 mg/kg		.40 (.37-.42)	.08 (—)
Control	2	2.8 (2.6-3.0)	.08 (—)
NaSCN, 75 "		.32 (—)	.08 (—)
Control	2	3.2 (3.1-3.3)	.10 (.09-.11)
$NaClO_4$, 15 "		.13 (.12-.14)	.11 (.10-.12)
Control	2	5.5 (4.3-6.7)	.09 (—)
Histamine, 3 mg		25 (23-27)	1.7 (1.5-1.9)
Male total innervated pouch dog			
Control	2	6.7 (6.4-7.0)	.19 (.15-.23)
NaI, 60 mg/kg		1.4 (1.1-1.6)	.15 (.12-.18)
Control	2	12 (9.4-14)	.27 (.25-.29)
NaI, 100 "		.90 (—)	.14 (.13-.15)
Control	4	4.3 (2.9-6.2)	.17 (.15-.19)
NaSCN, 75 "		.7 (.37-.80)	.16 (.09-.23)
Control	4	4.8 (3.4-6.8)	.20 (.12-.34)
$NaClO_4$, 15 "		.36 (.27-.54)	.21 (.13-.33)
Control	4	1.5 (1.0-2.7)	.24 (.17-.42)
Histamine, 3 mg		11 (9.0-13)	2.6 (1.7-3.3)

* Range; if no range stated there were 2 identical values.

kg). After a 30 minute period for mixing and equilibration, the pouch was washed and 2 15-minute experimental gastric collections made. Blood samples were taken at the mid-time of each. These specimens were analyzed for gastric clearance of chloride and I^{131} in the same manner as the samples from the acute experiments.

Results. Table I shows the control gastric I^{131} clearances varied among dogs and on the same dog from day to day. Control gastric I^{131} clearances varied from 1.5 to 11.7 cc plasma/min. The control gastric chloride clearances were 0.1 to 0.46 cc plasma/min., *i.e.*, 2% to 12% as great as the control iodide clearances. NaI administration depressed I^{131}

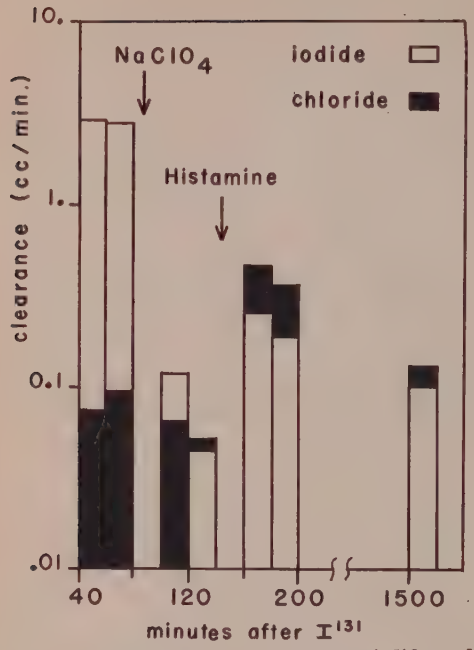

FIG. 1. Alterations in simultaneous iodide and chloride clearances of Heidenhain pouches. Each value represents an avg from 2 dogs. After each drug ($NaClO_4$ and histamine) one 20-min. collection was discarded without analysis.

clearance until it approached the chloride clearance. NaI did not cause as great a decrease in chloride clearance but there was the suggestion of a consistent reduction following iodide administration. NaSCN reduced gastric I^{131} clearance similar to NaI with no effect on chloride clearance except in the anesthetized dogs (Table I). $NaClO_4$ in 1/5 the dose of NaSCN produced the most severe reduction in I^{131} clearance which fell consistently to the level of chloride clearance. In these doses $NaClO_4$ produced no effect on chloride secretion. A single dose of perchlorate continued to exert maximum depression of gastric iodide clearance for more than 2 days in 3 dogs so studied.

Histamine produced a 10 to 18 fold increase in gastric chloride clearance in conscious dogs and 3 to 7 fold increase in the gastric I^{131} clearance.

In 2 dogs (Fig. 1) histamine was injected after demonstration of the $NaClO_4$ effect. The gastric chloride and iodide clearances then showed similar histamine responses.

Serpasil, used in 2 experiments on one dog, doubled the gastric I^{131} clearance and tripled the gastric chloride clearance. The Serpasil depressed the animal, and death occurred 3 days after the experiment. Diamox had no effect on basal chloride or I^{131} secretion.

Discussion. Apparently this is the first quantitative determination of the gastric I^{131} clearance in dogs. The results show: (1) those substances which greatly reduced the secretion of iodide by the gastric mucosa had little or no effect on the basal secretion of chloride, (2) histamine and reserpine produced a greater percent rise in the gastric chloride clearance than in iodide clearance, (3) the basal chloride clearance was only 2 to 12% of the basal iodide clearance, (4) when iodide secretion was blocked by a substance known to inhibit the thyroidal iodide trap, the gastric iodide clearance approached or reached the basal chloride clearance values. These data suggest that there are possibly two mechanisms for the secretion of iodide by the stomach, one that specifically traps iodide and rapidly secretes it, another non-specific mechanism in which iodide and chloride are interchangeable. This non-specific process was stimulated by histamine and not readily blocked. The specific process was effectively inhibited by elevated plasma iodide and by NaSCN or $NaClO_4$, similar to the iodide trap of the thyroid gland. Previous investigators who administered NaI probably blocked this specific iodide trap. The effect of $NaClO_4$, NaI, and NaSCN on the I^{131} clearance is possibly due to the anions of these salts combining with a specific system necessary for the transfer of iodide across the gastric cell. For normal function, this specific iodide trap may require an unsaturated carrier.

Histamine in absence of perchlorate (Table I) increased iodide clearance an average of 4 fold in unanesthetized dogs. Histamine in presence of perchlorate (Fig. 1) resulted in a similar (5 fold) increase in iodide clearance. Comparison of absolute results in gastric pouch dogs illustrates a dissimilarity between effects of histamine before and after

perchlorate. Before $NaClO_4$ injection, histamine increased the iodide clearance an average of 12 cc/min. but after $NaClO_4$, the increase was less than 1 cc/min. Therefore, the effects of histamine before and after perchlorate were relatively similar but absolutely quite different. This suggests that histamine had some effect upon the specific iodide trap of the stomach.

Summary. Iodide and chloride clearances were determined in anesthetized and unanesthetized dogs. The control gastric iodide clearance was 10-50 times greater than the chloride clearance. The iodide clearance could be 95% suppressed by anions known to block the thyroid iodide trap but chloride clearance was practically unchanged. In the presence of the blocking agent, gastric iodide and chloride clearances were similar. These data suggest at least 2 mechanisms for gastric secretion of iodide: (a) a specific iodide secretory system and (b) a non-specific mechanism in common with chloride secretion which continues to function after blockade of (a).

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Received July 23, 1956. P.S.E.B.M., 1956, v93.

Frequency of Abortive Parthenogenesis in Domestic Turkey.*† (22836)

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Abortive parthenogenesis in the chicken has been demonstrated to occur in eggs laid by virgin hens(1,2). Parthenogenesis also occurs in the turkey(3-5) and most of this development is of abnormal type. The available cytological evidence(6) is that nuclei of such parthenogenetically developing tissue are diploid. Kosin(7,8), in the absence of evidence to the contrary, ruled out parthenogenesis as a major factor responsible for the presence of aggregates of "moribund" cells observed in germ discs of apparently infertile eggs from mated hens. Following the first report by Olsen and Marsden(3) on the occurrence of parthenogenetic development in the turkey, a more intensive study of the matter was undertaken.

Materials and methods. The period reported here covers 1952-1956. The eggs were produced by the College strain of Broad Breasted Bronze hens and by U. S. D. A. strain of Beltsville Small White hens. The hens had been separated from males since 10-14 weeks of age. Upon separation, the birds were brought into completely enclosed pens where they remained for the entire observation period. Because the pens lacked windows, a low intensity artificial light (less than 1-foot candle power) was provided until the birds were 28 weeks old. At that time, the light intensity was increased to more than 5-foot candle power to stimulate oviposition.

* Scientific Paper No. 1515, Washington Agric. Exp. Stations, Pullman. Projects No. 803 and 1190.

† This investigation was supported, in part, by funds from Biological and Medical Research by State of Washington Initiative No. 171 and by Federal funds for regional research under the Hatch Amended Act, and conducted at Washington Agric. Exp. Station.

TABLE I. Frequency of Parthenogenetic Development in Broad Breasted Bronze Eggs.

	Unincubated		Incubated for 7 days	
Year	No. eggs	Positives, micro. examinations	No. eggs	Positives, micro. examinations
1954	101*	59 (58.4%)†	522	10 (2.8%)
1955			2594	65 (2.5%)

* These eggs received 10 hr of incubation.
† % of total eggs.

The 1953, 1954 and 1956 egg records were kept on pen-basis, while in 1955 trap nesting was introduced to identify the egg according to hen. Moreover, in the latter case there was an environmental difference: In one pen (No. 71), beginning in November, temperature was maintained at 55° ± 5°F; in the other pen (No. 70) the temperature fluctuated as before. The 1953 study, based on 1952 hatched birds, was designed primarily to develop suitable technics for assessing the prevalence of parthenogenesis in the domestic turkey. In 1954 the study was expanded to include observations on nearly 800 eggs. Some germ discs were studied histocytologically without first being subjected to incubation. Others were incubated for 7 days before being observed for gross signs of development. All 1955 and 1956 eggs were incubated for 7 days prior to examination. Discs which showed any evidence of tissue growth in the germ disc area were preserved for further histo-cytological study.

Results. The 1953 and 1954 material demonstrated that abortive parthenogenetic development occurs in the domestic turkey with higher frequency than that reported for the domestic chicken(1). Study of the 1954 eggs revealed that up to 80% of unincubated eggs laid by virgin turkey hens showed signs of parthenogenetic development. However, only a fraction of the germ discs showing such development on a cellular level, had the capacity to develop further (Table I).

In 1955 there was an indication of a difference in potential to develop parthenogenetically in the two groups subjected to different environments. In pen 70 (no temperature control) 3.5% of the eggs showed development; in pen 71 (temperature control), the corresponding figure was 2.2%. A complicating factor was introduced by the fact that birds in pen 70 developed a type of non-specific respiratory condition early in January, before egg laying began (trap-nesting was started on Jan. 23).

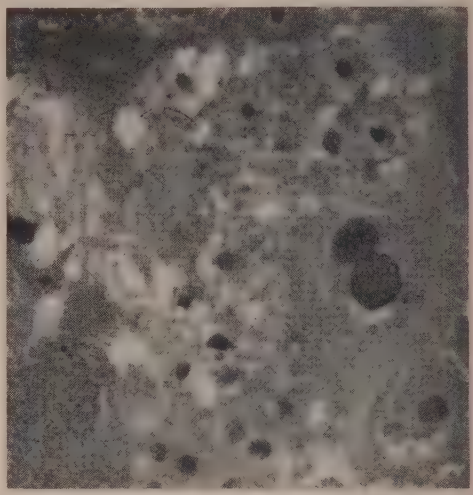

FIG. 1. Example of "single-cluster" type of cell proliferation in unincubated egg laid by virgin turkey hen.

FIG. 2 and 3. A more extreme type of development than shown in Fig. 1, frequently found in unincubated eggs from virgin turkey hens.

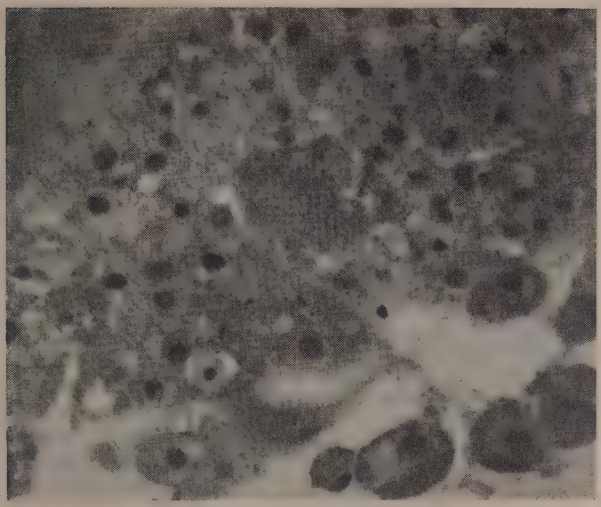

FIG. 3.

The unincubated turkey eggs which showed parthenogenetic development almost invariably were characterized by the presence of a single cluster of cells. In a number of instances, however, more than one such aggregate was observed in the blastodisc. (Fig. 1, 2, and 3.) The nuclei usually showed moderate affinity for basophilic stains, although only a few examples of active cell division have been encountered. This agrees with the observation that only a small portion of such germ discs are capable of developing further. In a great majority of cases, these cell clusters undoubtedly are either moribund cells or those which have recently died.

The observed level of abortive parthenogenesis in the Broad Breasted Bronze stock, as studied in this investigation, was considerably below that reported for Beltsville Small Whites(2,5). To check on this further, 100 eggs laid by virgin Beltsville Small White hens, and 100 eggs laid by mated Beltsville Small White hens of the strain which showed a high level of "positives" in the Beltsville work, were obtained from the Beltsville Research Center in late spring of 1955.‡ These B. S. W. eggs from virgin hens were incubated for 7 days and examined for evidence of "embryonic" growth. The eggs from the mated hens were used to provide an experimental flock of virgin Beltsville Small White hens raised under Pullman conditions.

Table II gives comparative figures on the frequency of parthenogenetic development in the eggs from 2 locations. There is obviously a genetic difference in the potential of the eggs to show such development. The location *per se* is not a major factor affecting the expression of that potential. Olsen and Marsden(2) have demonstrated that similar differences exist among the turkeys and chickens studied by them.

The fact that abortive parthenogenesis is of such common occurrence in turkeys poses certain problems. For example: Is the poten-

TABLE II. Population Differences in Frequency of Parthenogenesis following 7 Days of Incubation.

Year	Variety	No. of eggs	Positives, gross examination
1955	B. S. W.*	100	18.0
	B. B. B.	2954	2.9
1956	B. S. W.†	314	31.2
	B. B. B.	214	4.2

* Based on eggs obtained from U. S. D. A.
† Based on eggs laid by Beltsville Small White hens hatched in Pullman in 1955. 1956 data for both varieties include information up to June 6.

‡ The authors wish to acknowledge the cooperation of Drs. T. C. Byerly and W. A. Brant of the Agriculture Research Service, USDA, in providing these eggs.

tial for parthenogenetic development expressed to a greater or lesser degree in flocks of mated birds? Obviously, the answer to this question has a direct bearing upon the interpretation placed on finding cell colonies in so-called "infertile" eggs. Thus far, these usually have been considered to be instances of early embryonic mortality(8). Which of these "infertile" eggs from mated hens are developing parthenogenetically and which are cases of delayed development following fertilization? Olsen and Marsden(9) suggested that those turkey eggs from mated hens which show development only after 72 hours of incubation may be instances of parthenogenesis. Krizenecky *et al.*(10) approached the problem of morphological differences between infertile and non-fertile (from virgin hen) chicken eggs on the basis of the appearance of the germ disc. Unpublished data from this laboratory indicate that there may be similar differences in the turkey.

Another question is: Can the predisposition to parthenogenetic development serve as a deterrent to normal fertilization? It is conceivable that eggs possessing a high potential for such development would be less capable of being fertilized.

Summary. Evidence has been presented that: (1) Frequency of abortive parthenogenesis in the Broad Breasted Bronze turkey is as high as 80% when based on unincubated eggs studied histo-cytologically. (2) There are varietal differences which affect the frequency of occurrence of this type of development. Possibly, environmental differences also affect the frequency of abortive parthenogenesis.

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Received July 27, 1956. P.S.E.B.M., 1956, v93.

Effect of Anesthetics and Collection Time on Corticosteroid Secretion By Rat Adrenal.* (22837)

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Measurement of corticosteroids in the adrenal vein effluent of the rat has been described by Bush(1), Singer and Stack-Dunne (2) and Rosenman *et al.*(3). However, no attempt has been made to correlate secretion rate under these circumstances with time of collection of effluent and the nature of anesthetic employed. Several lines of evidence suggest that ether anesthesia is more stressful than Nembutal (pentobarbital sodium) and evokes a more profound pituitary-adrenocortical response than the latter(4-10).

The present observations support this conclusion and demonstrate that ether, but not Nembutal, anesthesia results in a marked early elevation of corticosterone in the ad-

* These investigations were aided by contract between Office of Naval Research, and University of California, NR 110-402, by grants G-1293 from National Science Foundation, and B-575 National Institute of Neurological Diseases and Blindness of N.I.H., Public Health Service.

renal venous effluent of the rat.

Methods. Adult, male Sprague-Dawley rats from our breeding colony were employed and maintained *ad libitum* on Purina laboratory chow and tap water. They weighed 300 to 500 g. Cannulation of the left renal vein was performed under ether or Nembutal anesthesia. Complete anesthesia was usually attained within 1 to 2 minutes with ether, and in approximately 15 minutes with Nembutal (50 mg/kg injected intraperitoneally). The technique of cannulation was similar to that described by Bush(1), as modified by Singer and Stack-Dunne(2), and was accomplished within 10 to 15 minutes after laparotomy. Heparin was used as an anti-coagulant. A series of timed collections was made of the left adrenal venous effluent in individual animals. The amount of blood collected was usually 2 to 4 ml for each time interval. Corticosteroids were extracted from blood samples by the method of Bush(1), as modified by Singer and Stack-Dunne(2). The extracts were then chromatographed on Whatman No. 1 filter paper employing toluene: methanol:water (4:3:1) system described by Bush(1). Known amounts of corticosterone and cortisol† were run as markers with each determination. Chromatographic development was allowed to proceed for 100 to 150 minutes at room temperature. Consistent results were obtained without temperature control. A complete change of the solvent phases within the chromatography cylinder was made only once every 3 to 4 months. Resolved steroids were located by spraying the air-dried filter paper sheets with alkaline Tetrazolium Blue‡ solution(11). The bulk of the corticosteroids in rat adrenal effluent invariably migrated in the corticosterone zone(1-3). Rarely, a trace of cortisol appeared as well(1). Elution of the stained steroids, as described by Touchston and Hsu(11), was carried out by immersing the dried chromatograms in distilled water for 3 minutes, followed by partial drying at room temperature. Sections of damp filter paper containing stained steroid were cut into small fragments and eluted twice with 5 ml of ethyl acetate:methanol (7:3). Each elution was for 10-15 minutes, with occasional shaking. Appropriate blank determinations were performed concurrently on unstained portions of the same chromatogram. These blanks usually had an absorbance of .02-.03, paralleling the average reading of .0269 reported by Touchston and Hsu(1). Photometric estimation of the dye eluted from the steroid-containing and control portions of chromatogram was made at 565 mμ in the Coleman Junior Spectrophotometer against a solvent blank set at zero density. Except for the 0 to 10 minute blood collections under Nembutal anesthesia, all density readings were equal to or greater than 0.05. The effective instrument error in this range is less than 10%. In the 0 to 10 minute Nembutal series only 1 reading was in this range. The value recorded for corticosterone secretion in this series (Table II) must therefore be considered maximal. *Quantitative chromatography* of pure corticosterone in amounts similar to those present in blood extracts yielded the results shown in Table I. The optical densities of the eluted dye followed Beer's Law (K = 0.50) over the range of 4 to 40 μg of corticosterone. The error inherent in these determinations averaged about 20% for concentrations of this steroid above 6 μg. In addition, recovery of corticosterone added to 2 ml of peripheral rat blood,

TABLE I. Photometric Analysis of Corticosterone Standards.*

Corticosterone, μg	No. of analyses	Optical density† (565 mμ)	Error, %‡	K§
4	15	.02 ± .002	30	.50
6	9	.03 ± .002	20	"
8	14	.04 ± .002	20	"
10	10	.05 ± .003	18	"
16	15	.08 ± .004	21	"
24	24	.12 ± .004	17	"
32	17	.17 ± .007	17	.53
40	10	.20 ± .010	17	.50

* Corticosterone was chromatographed, stained with Tetrazolium Blue and eluted with ethyl acetate-methanol (see text).
† Mean ± stand. error.
‡ Stand. dev./mean.
§ Mean optical density/μg corticosterone.

† Samples of pure corticosterone and cortisol were generously made available by Dr. Elmer Alpert, Merck and Co., Rahway, N. J.

‡ Nutritional Biochemicals Corp., Cleveland, O.

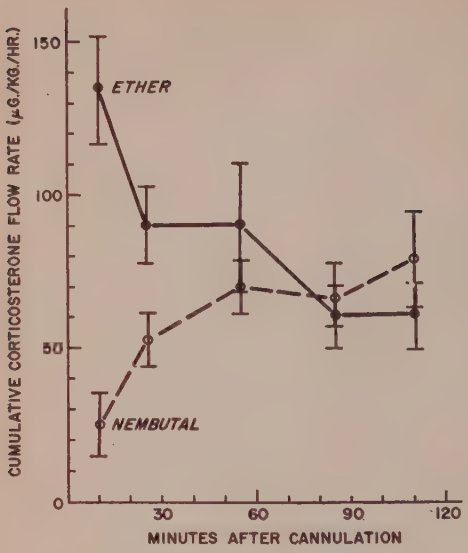

FIG. 1. Influence of anesthetic and collection time on cumulative corticosterone secretion rates of rat adrenal. Each point represents mean of 6 to 16 determinations, each on a different animal; stand. error is also shown.

and processed as described above, averaged 98% (90 to 104%) in 4 experiments with 50 μg of added steroid and 102% (88 to 144%) in 3 experiments with 10 μg of added steroid.

Results. The influence of time of collection and type of anesthetic on the amount of corticosterone appearing in the adrenal venous effluent of the rat following cannulation is depicted in Table II. Ether anesthesia resulted in elevated values for corticosterone concentration and corticosterone flow rate in the first 10 minutes of collection, as compared with corresponding values obtained after Nembutal anesthesia. This early difference was not evidenced in later collections (10 to 25 minutes and 25 to 85 minutes). However, cumulative values for rate of corticosterone release from the time of cannulation to the end of each collection period revealed that the differential effect of ether and Nembutal was apparent in collections made for 25 minutes or less, but not thereafter (Fig. 1).

It was not possible in the present investigations to dissociate the effects of anesthesia on corticosteroid release from those of the operative and collection procedures. It would appear likely, however, that the elevated early values observed after ether anesthesia were due, in part, to pituitary-adrenal activation associated with the administration of this anesthetic. A similar conclusion may be drawn from recent work on the effect of ether anesthesia on blood ACTH(4) and adrenal ascorbic acid levels(5) in the rat and on corticosteroid release by the dog adrenal (6). The use of ether as an anesthetic in studies on pituitary-adrenocortical function would appear to be contraindicated.

It is not certain whether the lower initial values for corticosterone release obtained after Nembutal anesthesia reflect the basal non-stressed corticosteroid output of the rat adrenal or partial inhibition of the early response to the stress of laparotomy. The latter possibility is supported by the observations of Sayers and Sayers(7), Ronzoni(8) and Cronheim and Hyder(9) on the inhibition by Nembutal of the adrenal ascorbic acid response to stress in the rat.

The similarity of corticosterone output values obtained during the later collection periods (Table II) under either Nembutal or ether anesthesia suggests that these later values represent the response of the rat adrenal cortex to the continued stresses of cannulation and blood loss. The maximal corticosterone concentrations observed in the present studies were 6.7 μg per ml of effluent with ether anesthesia and 7.0 μg per ml with Nembutal during the final collection period (25 to 85 minutes following cannulation).

The potentiating action of ether and the depressing effect of Nembutal on corticosterone secretion immediately following cannulation may be presumed to have been mediated via the hypothalamic-hypophyseal system. A central action of ether is supported by the observation of Sydnor and Sayers(4) that this anesthetic resulted in a rapid elevation of blood ACTH in the rat. However, a more direct effect of ether on the pituitary-adrenal system is not eliminated by these studies. The report by Cronheim and Hyder (9) that complete anesthesia with Nembutal blocked the response of the pituitary-adreno-

TABLE II. Effect of Anesthetic and Collection Time on Corticosterone Secretion by Rat Adrenal.

		Ether			Nembutal			
	Collection time, min.*	No. of rats	Vol of blood, ml†	Corticosterone secretion†	No. of rats	Vol of blood, ml†	Corticosterone secretion†	P value‡
Corticosterone flow rate (μg/kg/hr)	0-10	7	2.6 ± .4	136 ± 20	7	3.0 ± .8	25 ± 12	<.001
	10-25	6	2.0 ± .3	62 ± 10	5	1.8 ± .2	44 ± 24	.5
	25-85	11	3.8 ± .7	72 ± 15	16	4.4 ± .3	76 ± 12	.8
Corticosterone conc. (μg/ml)	0-10	7	2.6 ± .4	4.1 ± 1.0	7	3.0 ± .8	.9 ± .5	<.001
	10-25	6	2.0 ± .3	3.5 ± 1.2	5	1.8 ± .2	2.8 ± 1.3	.8
	25-85	11	3.8 ± .7	6.7 ± .8	16	4.4 ± .3	7.0 ± .9	.9

* Mean collection time. Collection times designated as 25 min. actually varied from 20 to 30 min.; those designated as 85 min. varied from 60 to 115 min.

† Values expressed as mean ± stand. error.

‡ Significance of differences between corticosterone secretion in Nembutalized *vs* etherized animals.

cortical system in the rat to salicylic acid suggests that this anesthetic exerts its primary action on the hypothalamus. The role of the hypothalamus in the activation of the pituitary-adrenal system is well established(12).

Cumulative corticosterone flow rates in the adrenal venous effluent of the rat obtained in the present study were somewhat lower than those reported by Singer and Stack-Dunne (2) and Rosenman *et al.*(3) under similar conditions. Variations in technic and age and strain of animals may be responsible for these differences. The considerably higher values reported by Bush(1) have been attributed, in part, to the administration of exogenous ACTH to his experimental animals. However, judging from the present investigations, the short collection time (10 to 20 minutes) under ether anesthesia may also have contributed to these high values.

Summary. Secretion of corticosterone into the adrenal venous effluent of the rat was markedly influenced during the first 10 minutes of collection by the nature of the anesthetic employed for cannulation procedure. Ether anesthesia resulted in a significant elevation of corticosterone concentration and corticosterone flow rate during this period as compared with the values observed when Nembutal was used. This difference was eradicated during subsequent collection periods of 10 to 25 minutes and 25 to 85 minutes following cannulation. The relationship of these findings to the possible activation by ether and inhibition by Nembutal of the pituitary-adrenocortical system is discussed.

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Received September 4, 1956. P.S.E.B.M., 1956, v93.

Conversion and Maintenance of *Histoplasma capsulatum* in Tissue Culture. (22838)

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The conversion of the diphasic fungus, *Histoplasma capsulatum,* to its tissue phase, has always been difficult, in our experience. However, many artificial media, such as a modification of Francis' cystine glucose blood agar (1), brain-heart infusion glucose blood agar and Salvin's synthetic medium(2), have been successfully used by many investigators. In recent years Kurung's egg(3), and Littman's liver-spleen media(4) were developed more or less specifically for the conversion and maintenance of *H. capsulatum* in its yeast phase. In addition to the artificial media Randall and his co-workers have advocated the use of tissue culture methods for the study of this fungus. They were successful in the *in vitro* cultivation of the tissue phase in media containing horse tissue, chicken fibroblast-like cells and the Earl's strain "L" mouse cells (5-7).

In preparation for studies on efficacy of antifungal agents it became evident that a more suitable, rapid and consistent method of obtaining the tissue phase of *H. capsulatum* had to be found. Therefore, the HeLa tissue culture method was explored in an effort to determine the efficiency of another animal material in which to study the fungus. Studies were made of the conversion and maintenance of the fungus with and without HeLa cells and its maintenance with and without rotation of the culture drum.

Methods and materials. The 13 isolates of *H. capsulatum* used in these studies were obtained from recent human cases. They were isolated from clinical specimens shipped to this laboratory. HeLa cells were cultivated in 250 ml bottles employing a medium composed of 20% human serum, 8% of a 1% yeast extract, 1% of a 20% glucose solution, 68% Hank's balanced salt solution (B.S.S.), 3% of a solution of 2.8% sodium bicarbonate and 0.001% phenol red. One hundred units of penicillin, 100 μg of streptomycin, and 0.2 μg of butyl parahydroxybenzoate were added to each ml of medium. The cells were trypsinized and stock solution of HeLa cells was made in HeLa maintenance medium composed of 63.5% HeLa mixture 199, 3% of a 5% sodium bicarbonate solution, 1% of a 20% solution of glucose, 25% tryptose phosphate broth and 7.5% calf serum. (Final pH of this medium was 7.4.*) No antibiotics were added. The cultures were initiated in 15 by 125 mm test tubes having bakelite caps and neoprene liners. Approximately 50,000 cells were suspended in each 1 ml of maintenance medium. Tubes were incubated at 37°C for 72 hours at a slant of 5°. After microscopic examinations were made of the tubes, usable ones were washed twice with phosphate buffered saline and replenished with 1 ml of maintenance medium. In attempting conversion of fungus to tissue phase a small amount of mycelium of each isolate was transferred to 1 ml of HeLa maintenance medium containing HeLa cells. These tubes were incubated, in a slanting position, at 37°C in the roller drum for 7 days. Microscopic examination was made at weekly intervals of growth, to determine degree of conversion. Then 0.1 ml of the culture was transferred, using sterile pipette, to a fresh tube of HeLa maintenance medium containing HeLa cells. This procedure was followed at the end of each week until isolates were completely converted to the yeast phase. The above procedure was followed in another experiment with the exception that the maintenance medium did not contain HeLa cells. It was of interest to learn whether HeLa cells were necessary to maintain *H. capsulatum* in its yeast phase. Accordingly, a series of tubes containing 1 ml of HeLa maintenance medium with HeLa cells and another series without HeLa cells were inoculated with a standard inoculum of yeast cells. The tubes were then placed in the roller drum and periodic read-

* We are indebted to H. A. Wenner, M.D., for this medium.

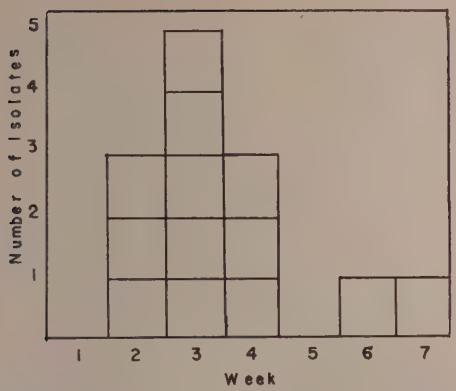

FIG. 1. Conversion of *Histoplasma capsulatum* to the yeast phase in the presence of HeLa cells. Illustrated are the No. of weeks required to convert 13 isolates in tissue culture medium.

ings made to determine amount and rate of growth. In addition to above experiments a study was made to determine the conditions necessary for propagation and maintenance of large numbers of yeast cells. Twenty ml of HeLa maintenance medium were dispensed into a series of sterile 100 ml centrifuge bottles with neoprene stoppers. Five isolates of the fungus previously converted to their yeast phase were selected. Five ml of a standard yeast inoculum were added to duplicate centrifuge bottles of each isolate. One set was placed in the roller drum; the other set remained stationary at 37°C. Daily readings were made to determine rate of growth and hydrogen-ion change of medium. Direct macroscopical observations of bottles were made to determine if rotation was necessary for maximum growth and maintenance of fungus in its yeast phase.

Results. Conversion to yeast phase with HeLa cells: In Fig. 1, the results of the conversion studies are presented. From these results it can be seen that there are differences in rate of conversion in HeLa maintenance medium from the original mycelial phase. At the end of 2 weeks incubation in roller drum, 3 isolates had completely converted to the tissue phase. Examination of several microscopical preparations from these tubes failed to reveal any fragments of mycelium. The greatest number of isolates (five) converted by the end of 3 weeks. Three additional isolates completely converted by the end of four weeks. The twelfth isolate converted by 6 weeks and the thirteenth reached complete conversion by the end of seventh week.

Conversion studies in absence of HeLa cells: Results of attempts at conversion to the yeast phase without HeLa cells are shown in Fig. 2. Six of the 13 isolates successfully converted completely to the yeast phase in the medium without HeLa cells. The first complete transition, 3 isolates, occurred by the end of fourth week. Four additional isolates converted, 2 in the fifth and 2 in sixth week of incubation. Six isolates failed to convert and very few, if any, yeast cells were seen in the microscopical preparation at the end of seventh week, when experiment was terminated.

Maintenance and propagation of yeast phase after conversion in presence and absence of HeLa cells: When the fungus has been converted completely to the yeast phase, HeLa cells are not absolutely necessary for propagation and maintenance of the culture. They do, however, enhance the rate of growth. In 4 of the 5 isolates studied, maximum growth occurred on the fourth day of incubation in medium containing HeLa cells. The

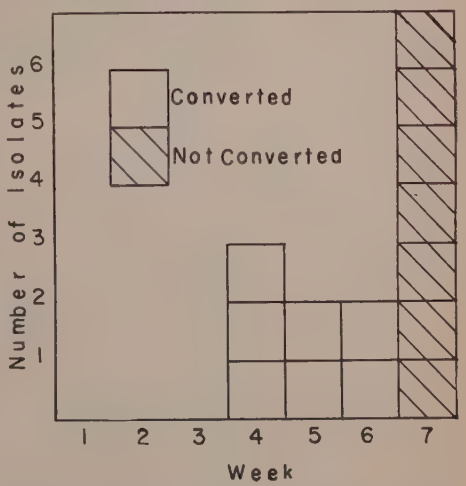

FIG. 2. Conversion of *Histoplasma capsulatum* to the yeast phase without HeLa cells. Illustrated are the weeks of transition for the 7 isolates which converted completely. Six isolates failed to convert by the end of 7 wk.

fifth isolate reached its maximum growth on sixth day.

In the medium without HeLa cells the same 5 isolates required 8 or 9 days to obtain comparable growth as reached in medium containing HeLa cells.

Determination of increase in numbers of cells by volumetric measurements showed an average increase of 30-fold. There was little difference between tubes containing HeLa cells and those without HeLa cells in the medium. In the small test tubes containing 1 ml of medium without HeLa cells there was an indication of reversion, in that a small amount of mycelium was found in 2 of the 5 isolates. During later studies in which large centrifuge bottles containing 20 ml of medium were used, no mycelium was detected in any cultures of the 5 isolates through 10 series of propagations.

Maintenance after conversion with and without rotation: The ultimate use of propagated yeast was to be in antifungal evaluation studies, which required large quantities of yeast cells. The amounts of yeast in small tubes were inadequate for experimentation. The successful conversion and maintenance of the yeast phase in medium without HeLa cells led to studies using 20 ml of medium in 100 ml centrifuge bottles with neoprene stoppers. This was the maximum amount of medium plus the quantity of inoculum that could be used in the slanting position of the roller drum.

The same 5 isolates showed a marked difference in rate of growth in the 100 ml centrifuge bottles when subjected to rotation or held stationary. Four of the 5 isolates reached maximum growth on fourth day of rotation, the fifth isolate on the fifth day. None of the bottles maintained in stationary position in the same incubator reached a comparable growth at end of 10 days.

Discussion. In our experience the tissue culture method has proved very satisfactory for conversion and maintenance of *H. capsulatum* in the yeast phase. It is apparent from Figs. 1 and 2 that presence of HeLa cells in the medium has a marked influence upon rate and final outcome of the yeast phase transition. The ease with which 13 isolates of fungus converted to the tissue phase suggests that the method is rapid and reproducible. Large quantities of cells can be produced by this technic. It has the advantage that the yeast phase can be readily propagated in a liquid medium by weekly transfers. After acclimation to a liquid menstruum and using the roller drum method, these cells can be easily incorporated in antifungal studies.

In addition to *H. capsulatum* this method has proved satisfactory in preliminary studies, for conversion and maintenance of *Blastomyces dermatitidis, Sporotrichum schenckii* and *Coccidioides immitis.* The last named fungus was most difficult to convert to the tissue phase. Data available at this time suggest that the ratio of medium to inoculum is critical. Too much mycelium tends to prevent formation of large numbers of spherules. It is of significance that copious mycelium developed at 37°C when small pieces of infected liver or spleen tissues from mice were inoculated into HeLa maintenance medium. Very few spherules were observed in the first transfer from infected tissue. The ease of mycelium production at blood temperature in a defined liquid medium may be correlated with the frequent reports of mycelium in lungs infected with *C. immitis.*

Candida albicans and *Cryptococcus neoformans* were easily propagated in tissue medium without HeLa cells.

The results of these investigations stimulated further use of tissue culture methods in evaluating antifungal agents against systemic fungi. The technic lends itself very well to evaluation of the effects of the antifungal agent on HeLa cells and tissue phase of the fungus.

It is recognized that tissue culture method is expensive and requires that personnel be proficient in the technic. However, the widespread adoption of the method in virology and other studies has made this laboratory tool available in most research institutions.

Conclusions. 1. Tissue culture method can be successfully used to convert *Histoplasma capsulatum* to the tissue phase. 2. Six of 13 isolations did not convert to the tissue phase in absence of HeLa cells; with all isolates conversion was more complete and rapid in

presence of cells. 3. The yeast phase can be propagated and maintained in tissue culture mediums without HeLa cells in 100 ml centrifuge bottles. 4. Rotation of cultures is necessary to assure maximum growth of yeast cells in tissue culture medium. 5. The technic can be successfully used to convert other diphasic systemic fungi, *Blastomyces dermatitidis, Sporotrichum schenckii,* and *Coccidioides immitis* to their tissue phase. 6. *Candida albicans* and *Cryptococcus neoformans* can be propagated in tissue culture medium without HeLa cells.

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Received October 4, 1956. P.S.E.B.M., 1956, v93.

Effect of *Rauwolfia serpentina* on Thyroid and Pituitary Glands of Young Rats.* (22839)

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The active components of *Rauwolfia serpentina* are held to exert their effects by way of the central nervous system, and it is believed that their influence on the endocrine glands is of minor consequence (Gaunt *et al.* 1). However, in the present study the prolonged administration of a preparation of the whole root to a group of young rats resulted in a significant diminution of the weights of both the pituitary and thyroid glands.

Procedure. The rats employed were of the Sprague-Dawley strain, females, and 21 days of age at the beginning of the experiment. Twenty were treated and 16 littermates remained uninjected as controls. The *Rauwolfia serpentina* was given in aqueous solution by subcutaneous injection and at a daily dosage equivalent to 1 mg per kilo body weight. A fresh solution was prepared and adjusted once weekly to the weight of the animals. The injections were continued until the animals were from 65 to 70 days old when they were sacrificed with illuminating gas. At autopsy the body weight and the weights of the hypophysis, thyroid, adrenals, and ovaries were obtained.

* Supported in part by research grant No. G4034 from N.I.H., U.S.P.H.S.

Results. The rats remained active and in good condition throughout the period of treatment, although the injected animals ate somewhat smaller quantities of food and eventually their body weight was generally less than that of the controls. The day of vaginal opening, indicating the onset of complete ovarian function, occurred from the 43rd to the 52nd day of age in the experimental group as compared to the 41st to the 46th day in the controls.

The organ and body weights of the individual rats found at autopsy are given in Table I, while Fig. 1 presents the data for the thyroid and pituitary weights arranged on a semi-logarithmic scale. The oblique lines (broken lines for the controls; continuous for the experimental) represent the average log-organ to body weight relationship as estimated by the method of least squares. The vertical distance between the two lines in each case offers comparison between the organ weights with allowance for body weight. For the pituitary gland this vertical difference is .0338 (in units of logarithms) which corresponds to an 8% smaller size (after adjustment for body weight) in the experimental than in the control group. This dif-

TABLE I. Body and Organ Weights of Rats following Daily Injection of *Rauwolfia serpentina* 1 mg per Kilo Body Weight for 44 to 49 Days.

Experimental					Controls				
Body wt	Adren.	Ovaries	Thyroid	Pit.	Body wt	Adren.	Ovaries	Thyroid	Pit.
(g)									
146	.041	.041	.006	.0065	170	.047	.049	.011	.009
168	.045	.056	.0065	.0085	169	.051	.052	.010	"
180	.046	.050	"	"	174	.045	.047	.011	"
157	.039	.053	"	"	184	.059	.046	"	.010
156	.051	.051	"	.008	186	.048	.072	"	.011
171	.054	.056	.007	.0085	188	.053	.063	.010	.010
186	.042	.059	.008	.0095	196	.054	.057	.011	.011
188	.060	.066	.0075	"	201	.058	.069	"	"
153	.041	.048	"	.0075	197	.049	.058	"	"
197	.053	.046	"	.0095	184	.052	.051	"	.010
200	.060	.066	.0085	.010	166	.048	.050	"	"
134	.048	.020	.0065	.0075	180	.054	.046	"	"
180	.045	.055	.008	.0095	170	.048	.024	.010	.008
184	.055	.054	"	"	190	.068	.061	.011	.010
165	.042	.031	.0075	.008	184	.070	.074	"	.011
158	.040	.047	.007	.0075	189	.065	.071	.010	"
190	.051	.054	.008	.0095					
191	.046	.065	"	.010					
188	.055	.062	.009	"					
174	.052	.055	.0085	.009					

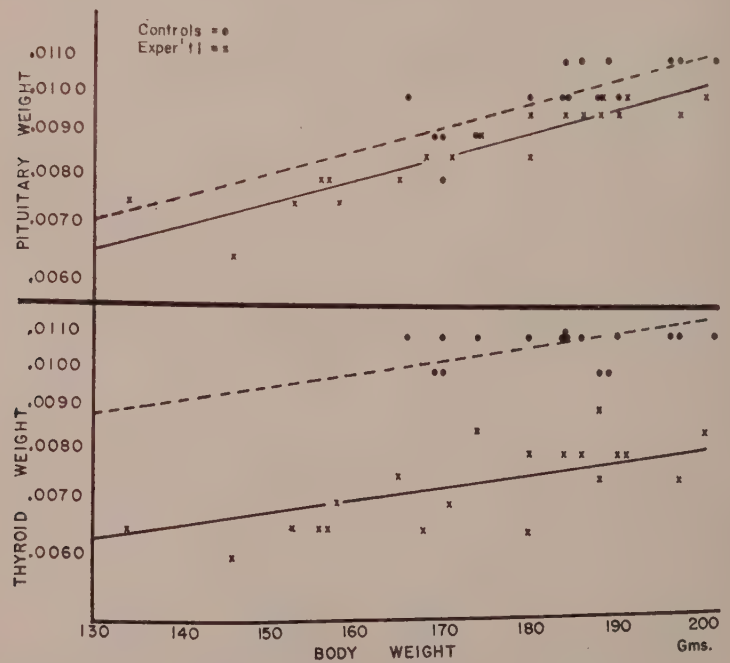

FIG. 1. Oblique lines (broken lines for controls, continuous for experimental) represent avg log-organ to body wt relationship as estimated by the method of least squares.

ference has a standard error of .0126 and is thus statistically significant at the 1% level.

The corresponding difference for the weights of the thyroid is .1467 (standard error of .01), corresponding to a 40% smaller weight in the experimental group than in the control. The data for the ovaries and the adrenals show no effect when analysed statistically by the same method (analysis of covariance).

Administration of *Rauwolfia serpentina* at daily dosages of 2, 3, and 6 mg per kilo body weight and for similar periods of time resulted in such a profound loss of body weight that it was felt that organ differences were greatly affected by changes due solely to partial starvation. Another group of 20 rats was treated in the same manner with a daily dosage of 2.5 mg per kilo body weight of chlorpromazine (Thorazine—S.K.&F.) but without any appreciable change in body and organ weights.

Summary. 1. An extract of *Rauwolfia serpentina* was administered daily by subcutaneous injection to 20 rats. The daily dosage was equivalent to 1 mg/kilo body weight and was continued from 21st to the 65th to 70th days of life. At autopsy body weights and weights of pituitary gland, thyroid, ovaries, and adrenals were obtained. 2. Application of analysis of covariance leads to the conclusion that at this dose level administration of *Rauwolfia serpentina* to young rats resulted in (1) a diminution of weight of thyroid by 40%, at the 1% level; (2) a similar reduction in weight of pituitary gland by 8%; (3) no significant change in either ovarian or adrenal weights, after making allowance for body weight differences.

My thanks are due to Dr. Lincoln E. Moses for statistical analyses; to Mr. V. Montesclaros for technical assistance; to Dr. Georg Cronheim and Riker Laboratories for *Rauwolfia serpentina* and to Dr. William E. Kirsch and Smith, Kline and French for the chlorpromazine.

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Received October 19, 1956. P.S.E.B.M., 1956, v93.

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